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## Multidrug resistance proteins (MRP) transport the cytokine/nuclear protein High Mobility Group Box 1 (HMGB1) across membranes

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**Elisa Latorre**

**Multidrug Resistance Proteins (MRP)  
transport  
the cytokine/nuclear protein  
High Mobility Group Box 1 (HMGB1)  
across membranes**

**THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT OF THE OPEN  
UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MOLECULAR AND  
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## **List of abbreviations**

ABC: ATP-Binding-Cassette  
ANOVA: analysis of variance  
ATP: Adenosin-5'-triphosphate  
BSA: bovine serum albumin  
BSO: L-buthionine-sulfoximine  
CD14: cluster of differentiation 14  
DAPI: 4', 6-diamidino-2-phenylindole  
DC: dendritic cell  
DHFR: dihydrofolate reductase  
DIDS: 4,4' diisothiocyanatosilbene-2,2'- disulfonic acid disodium salt  
DMEM: Dulbecco's modified Eagle's medium  
DTT: dithiothreitol  
ER: endoplasmic reticulum  
ERK: extracellular-signal Regulated Kinase  
ETA: Ethacrynic Acid  
GCCL : glutamic acid/cysteine ligase  
GFP: green fluorescent protein  
Gly: Glybenclamide  
GSH: reduced glutathione  
GSSG: oxidised glutathione  
HMGB: high mobility group box  
IEM: immune electron microscopy  
IL-1: interleukin 1  
IL1beta: Interleukin 1 beta  
IL-1RA: IL-1 receptor antagonist  
KO: knock out  
LB: Luria-Bertani broth  
LBP: LPS binding protein  
LEF-1: lymphocyte enhancer factor 1  
LPC: lysophosphatidylcholine  
LPS: lypopolysaccaride  
MDR1: multiple drug resistance transporter  
MHC: major histocompatibility complex

**MIF:** macrophage migration inhibitory factor  
**MK571:** (E)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3  
 oxopropyl]thio]methyl]thio]-propanoic acid  
**MRP1:** multiplerdrug resistance protein 1  
**MTX:** methotrexate  
**NBF:** nucleotide binding folds  
**NES:** nuclear export signal  
**NLS:** nuclear localization singal  
**PBS:** phosphate-buffer saline  
**PCR:** polymerase chain reaction  
**PFA:** paraformaldehyde  
**PMA:** Phorbol 12-myristate 13-acetate  
**Prob:** Probenecid  
**RAGE:** receptor for advanced glycation end-products  
**SDS:** sodium dodecyl sulphate  
**SDS-PAGE:** SDS polyacrylamide gel electrophoresis  
**SOX:** SRY related homeobox  
**SRY:** sex-determining region Y  
**THIO:** Thioredoxin  
**TLR:** toll like receptor  
**TMD:** two transmembrane domains  
**TSA:** trichostatin A  
**Vera:** Verapamil  
**WB:** western blotting  
**WT:** wild type

## Abstract

High mobility group box 1 (HMGB1) is a mobile chromatin protein that can relocate into the external environment and act as a cytokine. HMGB1 cellular release can be passive, by cell necrosis, or active, by living cells after a specific stimulus is received.

HMGB1 does not possess a leader sequence, a sequence usually present at the N-terminus of secreted proteins that is sufficient to address them through the ER/Golgi exocytic pathway. This work focused on the molecular mechanism by which HMGB1, as a leaderless protein, can be actively exocytosed by living cells.

HMGB1 undergoes post-translational modifications that allow it to move to various cellular sub-compartments:

- 1) Translocation from the nucleus to the cytosol
- 2) Loading inside exocytic vesicles (secretory lysosomes)
- 3) Release into the extracellular milieu by vesicle degranulation.

The post-translational modification involved in the first translocation step was already known to be lysine acetylation. Here I demonstrate the necessity of cysteine glutathionylation for the translocation inside secretory lysosomes, and the direct involvement of Multidrug Resistance Proteins (MRP/ABCC) in the transport process.

I started with the observation that HMGB1 and IL-1 $\beta$ , another leaderless cytokine, do colocalize inside secretory lysosomes in macrophages, and that a pharmacological inhibitor of IL-1 $\beta$  exocytosis, glybenclamide, inhibits HMGB1 release too. Glybenclamide is a general inhibitor of the ABC transporter Superfamily, a large family of transmembrane proteins that transport ions, drugs and small peptides. Based on the sensitivity or insensitivity to different drugs, the HMGB1 transporter was likely to belong to ABC C group.

Overexpression of MRP1/ABCC1, MRP2/ABCC2 and MRP3/ABCC3 endows activated cells with the ability to secrete HMGB1. Moreover, macrophages from Mrp1<sup>-/-</sup> mice have impaired HMGB1 exocytosis. I also suggest that HMGB1 is unfolded during its translocation.

MRP transporters are known to recognize glutathionylated substrates. Indeed, HMGB1 can be glutathionylated, and depletion of the glutathione intracellular pool impairs HMGB1 secretion.

MRP transporters can be overexpressed in drug resistant tumour cells, where they pump chemotherapeutic agents out of the cell. I suggest that cells that are drug-resistant because of MRP1 overexpression might also secrete HMGB1.

## Introduction

### HMGB proteins (High Mobility Group Box) and HMGB1

The HMGB proteins, so-called for 'high mobility group', are perhaps the most extensively studied non-histone chromosomal proteins. They comprise 3 families:

- 1) HMGB (HMGB1/2) family with molecular mass of about 25 kDa
- 2) HMGN (HMGB14/17) family with molecular mass of about 10 kDa
- 3) HMGA (HMGB- I/Y/C) family with molecular mass of about 15 kDa

HMGB proteins in mammals comprise HMGB1, HMGB2 and HMGB3. The structure of these proteins is highly conserved (more than 80% amino acid sequence identity), and their biochemical properties are so far indistinguishable. HMGBs are composed of 3 different domains: two homologous DNA binding domains, HMGB-box A and HMGB-box B, which are each around 75 amino acids long, and the C-terminus domain which is highly negatively charged and consists of a stretch of glutamate and aspartate residues. The acidic tail modulates DNA binding properties of HMGB1 (Knapp et al 2004), and is shorter in HMGB2 and shortest in HMGB3. The main ability of HMGB-boxes is to bind DNA in the minor groove and produce distortions in the double helix.

Putative counterparts of mammalian HMGBs are present in:

*S. cerevisiae* (non-histone proteins 6A and 6B, NHP6A and NHP6B)

*D. melanogaster* (HMG-D and HMG-Z)

all vertebrates, including *X. levis* (x-HMG) and zebrafish (6 proteins).

Single HMGB-box domains, with no acidic tail, are characteristic of transcription factors like SRY (sex-determining region Y), SOX (SRY-related HMGB-box) and LEF-1 (lymphocyte enhancer factor 1), the nucleolar transcription factor UBF (upstream binding factor), the lymphoid transcription factors TCF1 (T-cell factor 1) and the yeast mating genes *mat-Mc* and *MATa1* (Bustin et al 2002; Thomas and Travers 2001).

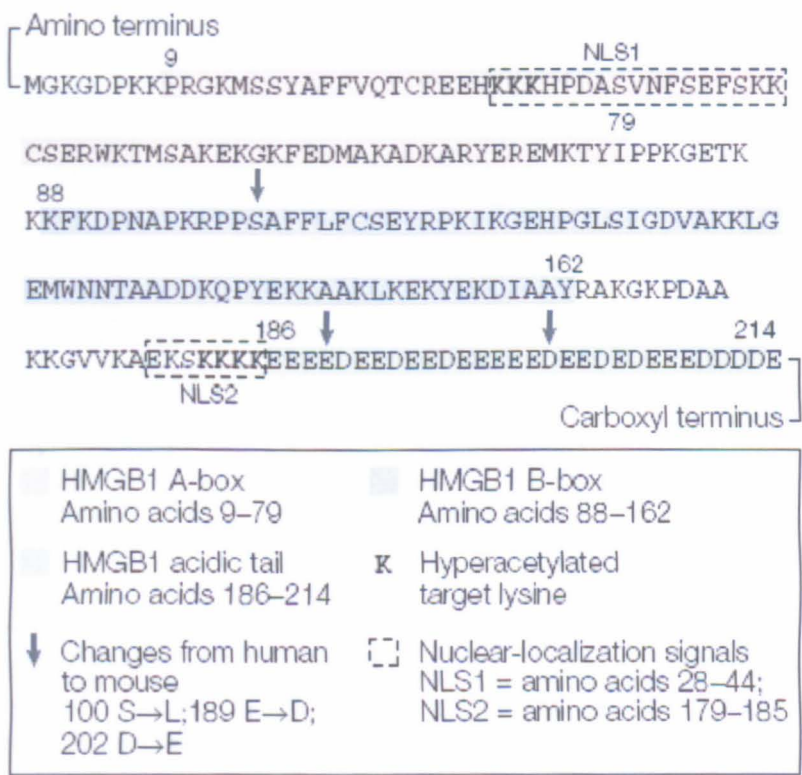
### HMGB1 protein

The non-histone nuclear-binding protein high-mobility group box 1 protein (HMGB1), is encoded on human chromosome 13q12–13. It is a small protein, approximately 28 kDa, and is an abundant component of all mammalian nuclei.

The entire human HMGB1 cDNA was sequenced by Wen (1989), from a human placenta cDNA library. Northern blot analysis showed that 3 mRNA species of approximately 1.0, 1.4, and 2.4 Kb were expressed in all mammalian organs and cell lines examined.

As mentioned before, the protein is able to bind with high affinity to specific DNA structures and bend them, but does not show nucleotide sequence preference (Bianchi et al 1989). In the beginning, HMGB1 was considered and studied for its chromatin binding capability and as a chromatin structural protein. HMGB1, in the nucleus, interacts with the DNA minor groove and several nuclear transcription factors, acting as a transcriptional chaperone. Later it was found that, when it is present in the cell external environment, HMGB1 behaves in a cytokine-like fashion (Wang et al 1999).

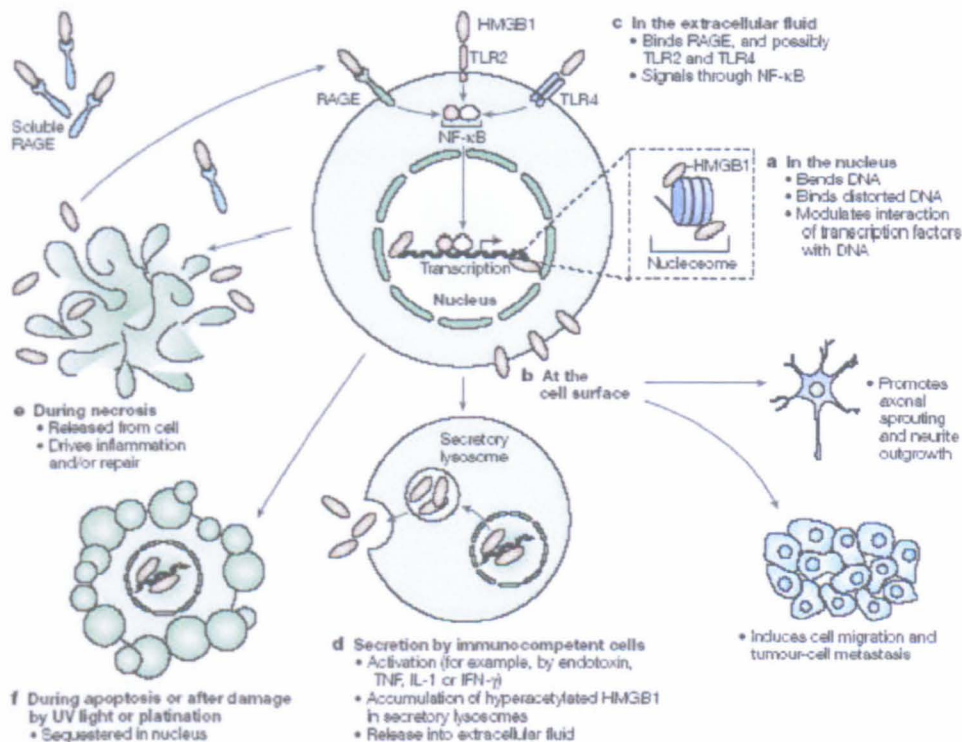
The amino acidic stretch displays 2 nuclear localization signals (NLS1 and NLS2) but no secretion leader sequence is present. Moreover, HMGB1 contains several acetyltable lysine residues, as depicted in figure 1 (Muller et al 2001, Lotze and Tracey 2005).



**1) Linear diagram of HMGB1** (Lotze and Tracey 2005) The diagram includes the residues that constitute the A-box (pink), B-box (purple) and acidic tail (green). HMGB1 also contains 43 lysine residues, some of which are frequently acetylated in lipopolysaccharide-activated macrophages (shown in bold). These lysine residues are found within two nuclear-localization signals (indicated by dashed boxes): NLS1, which spans amino acids 28–44; and NLS2, which spans amino acids 179–185. The acidic carboxyl terminus contains two amino acids that differ between mice and humans (indicated by arrows). This acidic region is thought to interact with and protect the A-box and B-box during emigration from the nucleus.

HMGB1 is passively released from necrotic cells and actively secreted by activated myeloid cells and tumour cells. As a cytokine, HMGB1 activates endothelial cells, promotes

angiogenesis, enhances haematopoietic stem-cell migration and drives inflammation (Degryse et al 2001, Schlueter et al 2005). HMGB1 endothelial stimulation leads to enhanced expression of adhesion molecules, including intercellular adhesion molecule 1, vascular cell-adhesion molecule 1, and RAGE (receptor for advanced glycation end-products), see figure 2 (Lotze and Tracey 2005).



## 2) Intracellular and extracellular roles of HMGB1 (Lotze and Tracey 2005)

**A.** HMGB1 in the nucleus interacts with the nuclear matrix, chromatin and transcription factors; it functions as a transcriptional chaperone.

**B.** HMGB1 present at the cell surface promotes axonal sprouting and neurite outgrowth. Membrane-bound HMGB1 also promotes cell migration and tumour metastasis.

**C.** In the extracellular fluid, HMGB1 signals through RAGE (receptor for advanced glycation endproducts), and possibly through Toll-like receptor 2 (TLR2) and TLR4,

**D.** HMGB1 can be secreted by activated macrophages and dendritic cells, after activation by bacterial products, such as endotoxin, or pro-inflammatory cytokines

The secretion pathway involves nucleus-cytosol translocation of hyperacetylated HMGB1, loading in secretory lysosomes, fusion with the cell membrane and release into the extracellular milieu.

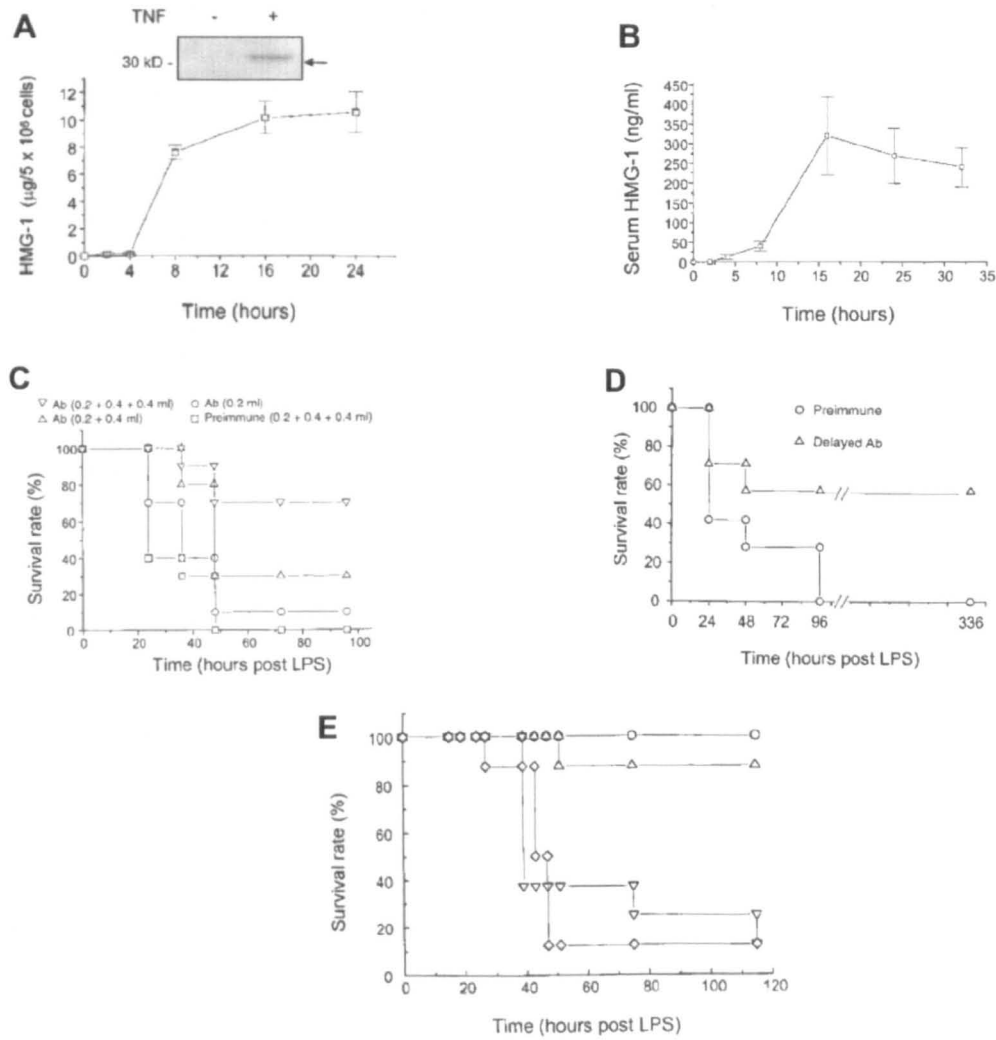
**E.** Necrotic cell release HMGB1 is by not the tightly bound histones.

**F.** During apoptosis HMGB1 is sequestered by the chromatin.



In 1999 Wang first identified HMGB1 as a potential late mediator of lethality due to bacterial endotoxin (LPS). HMGB1 protein was found to be released by cultured macrophages more than 8 hours after stimulation with LPS, TNF-alpha or IL-1beta. Mice showed increased HMGB1 serum levels after the treatment. Administration of antibodies against HMGB1 attenuated LPS lethality, and administration of HMGB1 itself was lethal (figure 3).

Wang also studied HMGB1 release in human serum, and he observed that in normal patients the protein was not detectable, but significant levels were observed in patients with critical septicaemia. HMGB1 levels were higher in patients that died, if compared with patients that displayed non lethal infections. (Wang et al 1999; Lotze and Tracey 2005).



### 3) HMGB1 and sepsis (Wang et al 1999)

#### A. Release of HMGB1 from cultured macrophages after stimulation with LPS

Murine macrophage-like RAW 264.7 cells were treated with LPS (100 ng/ml) and proteins in the cell-conditioned medium were analysed by western blotting with anti-HMGB1.

#### B. Accumulation of HMGB1 in serum of LPS-treated mice

Male Balb/C mice (20 to 23 g) were treated intraperitoneally (ip) with 10 mg/kg LPS. Serum was assayed for HMGB1 by western blotting.

#### C. Anti-HMGB1 antibodies protect against LPS lethality in mice

Male Balb/C mice (20 to 23 g) were randomly grouped (10 mice per group) and treated with LPS at LD<sub>100</sub> (25 mg/kg). Anti-HMGB1 (Ab) or preimmune serum (0.2 ml per mouse, ip) was administered 30 min before LPS. Additional doses of preimmune (0.4 ml, ip) or anti-HMGB1 (0.4 ml, ip) were administered at 12 and 36 hours after LPS as indicated.

#### D. Delayed administration of anti-HMGB1 protects against LPS lethality in mice

Male Balb/C mice (20 to 23 g) were randomly grouped (seven mice per group) and treated with LPS at LD<sub>100</sub> of. Anti-HMGB1 or preimmune serum (0.4 ml per mouse) was administered at 2, 24, and 36 hours after LPS.

#### E. Administration of rHMGB1 is lethal to mice

Male Balb/C mice (20 to 23, 10 animals per group) were injected with a non lethal dose of LPS (3.1 mg/kg, ip). Purified rHMGB1 protein was administered intraperitoneally in the doses indicated at 2, 16, 28, and 40 hours after LPS.

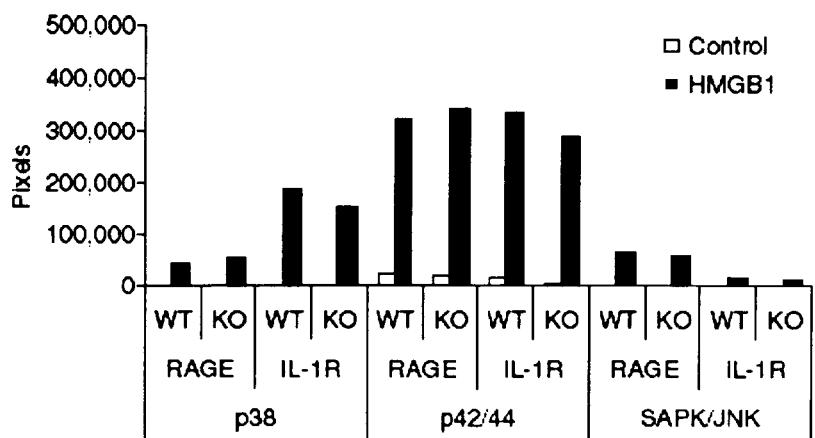
In 2004 Yang et al reported that, in a murine sepsis model of surgically induced peritonitis, HMGB1 serum levels are significantly increased. Specific inhibition of HMGB1 activity, by antibodies against HMGB1 or HMGB1 DNA-binding A box, significantly increased mice survival. Animals pre-treated with either HMGB1 antagonist were protected against the development of sepsis-induced multi-organ injury. These observations demonstrated that the specific inhibition of endogenous HMGB1 therapeutically reverses the lethality of induced sepsis.

Interestingly, in 2002 Zetterstrom identified HMGB1 as an antibacterial factor produced and stored intracellularly in human adenoid glands.

### HMGB1 receptors

A great deal of evidence indicates RAGE (Receptor for Advanced Glycation Endproducts) as HMGB1 cell surface receptor. Therefore, colocalization of RAGE and HMGB1 on the leading edge of advancing neurites and glioma cells, indicates their potential contribution to cellular migration and tumour invasion (Parkkinen et al 1993, Lotze and Tracey 2005).

In 2000 Taguchi demonstrated that blockage of RAGE/HMGB1 interaction decreases growth and metastases of both implanted tumours and tumours developing spontaneously in susceptible mice. Inhibition of the RAGE/HMGB1 interaction suppressed activation of p44/p42, p38, and SAP/JNK MAP kinases, and expression of matrix metalloproteinases.



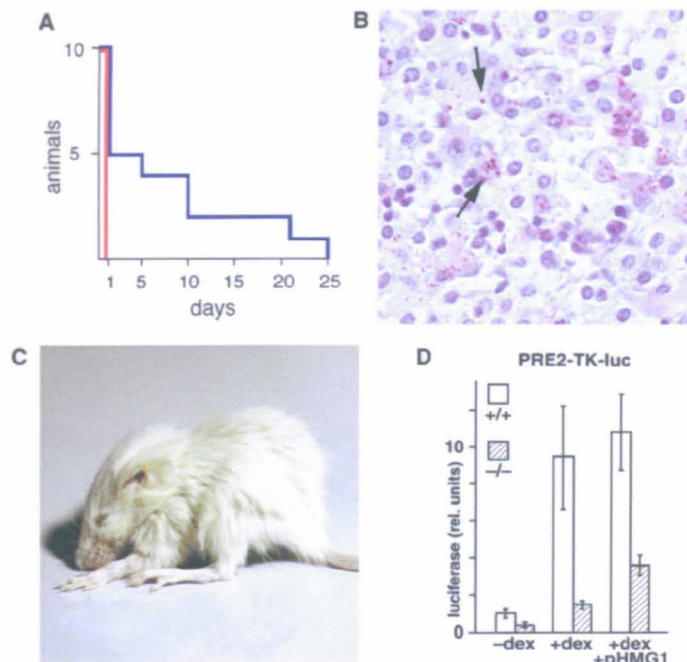
**4) RAGE is not the unique HMGB1 receptor (Kokkola et al 2004)**

HMGB1 induces mitogen-activated protein (MAP) kinase phosphorylation in RAGE<sup>-/-</sup> and IL-1R<sup>-/-</sup> mouse macrophages (Mf). Mf derived from RAGE<sup>-/-</sup> and IL-1R<sup>-/-</sup> mouse bone marrow were stimulated with HMGB1 (10 mg/ml) or medium alone (control) and the phosphorylation of p38 MAPK, p44/42 MAPK and SAPK/JNK was recorded after 30 min by western blotting using antibodies against the phosphorylated forms of MAP kinases. The grade of phosphorylation is expressed as net intensity.

Kokkola in 2004 reported that macrophages from RAGE<sup>-/-</sup> mice produce significantly lower amounts of TNF, IL-1b and IL-6, while IL-1R<sup>-/-</sup> and TLR2<sup>-/-</sup> macrophages produce cytokine levels comparable with wt controls in response to HMGB1 stimulation. Kokkola concluded that HMGB1 has the potential to induce a pro-inflammatory phenotype by acting mainly throughout RAGE, but other minor receptors and transduction pathway must exist (Figure 4).

**Phenotype of HMGB1- and HMGB2-deficient mice**

Calogero et al (1999) generated Hmgb1 conventional KO mice. Hmgb1<sup>-/-</sup> pups are born alive, but die within 24 hours because of hypoglycaemia.



### 5) *Hmgb1*<sup>-/-</sup> mice develop neonatal hypoglycaemia (Calogero et al 1999)

**A.** *Hmgb1*<sup>-/-</sup> mice die within the first day of life but can survive if given glucose parenterally. Intraperitoneal glucose injections were administered to 10 *Hmgb1*<sup>-/-</sup> newborns during the first days after birth. Survival is indicated by a blue line. Ten *Hmgb1*<sup>-/-</sup> control mice, injected with saline solution, all died within day 1 (red line), similar to untreated *Hmgb1*<sup>-/-</sup> mice.

**B.** Periodic acid-Schiff (PAS) staining for glycogen (magenta) in livers from *Hmgb1*<sup>-/-</sup> mice are shown. Despite very low blood glucose concentrations, hepatocytes of *Hmgb1*<sup>-/-</sup> mice do not completely mobilize glycogen (arrows, glycogen granules).

**C.** Phenotype of an *Hmgb1*<sup>-/-</sup> spontaneous survivor at day 25. Spontaneous survivors of the mixed 129Sv/CD1 genetic background are very similar to the most successfully glucose-rescued *Hmgb1*<sup>-/-</sup> mice of 129Sv/BALB-c background: they have a very reduced size, but respond positively to basic neurological tests. All spontaneous or glucose-treated surviving animals have sealed eyelids, arched backs, long hind paws and abnormal gait.

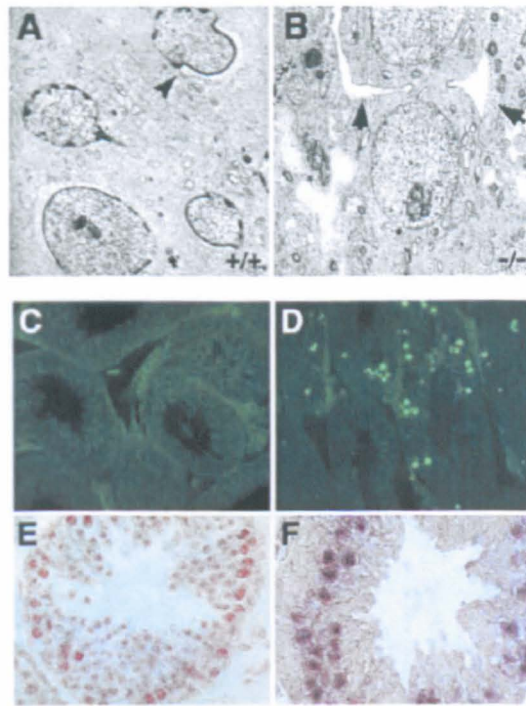
**D.** The absence of Hmgb1 reduces the activity of GR in transfection assays. The expression of a GR-controlled reporter is reduced in *Hmgb1*<sup>-/-</sup> fibroblast cell lines exposed to dexamethasone.

*Hmgb1* KO mice survive for several days, if glucose solution is injected intraperitoneally, then waste away with pleiotropic defects (with no alteration in the immune repertoire) (Figure 5A, C).

It was observed that a high glycogen level is present in the liver (figure 5B) but is not metabolized. Cell lines lacking Hmgb1 grew normally, but the gene expression controlled by

the glucocorticoid receptor (GR) is impaired (figure 5D). Calogero et al concluded that Hmgb1 is not essential for the overall organization of chromatin in the cell nucleus, but is critical for proper transcriptional control by specific transcription factors.

Ronfani et al (2001) generated Hmgb2<sup>-/-</sup> mice; these were healthy but males had reduced fertility. This phenotype was due to an increased apoptosis level (Figure 6) in seminiferous tubules, resulting in a diminished production of spermatozoa. HMGB2 protein does not seem to be essential, perhaps HMGB1 has a redundant function.



#### 6) Abnormalities in the testis of *Hmgb2*<sup>-/-</sup> mice (Ronfani et al 2001)

**A. B.** Electron micrographs of an elongated spermatid where the acrosome (arrows) is detached from the nucleus, impairing its function.

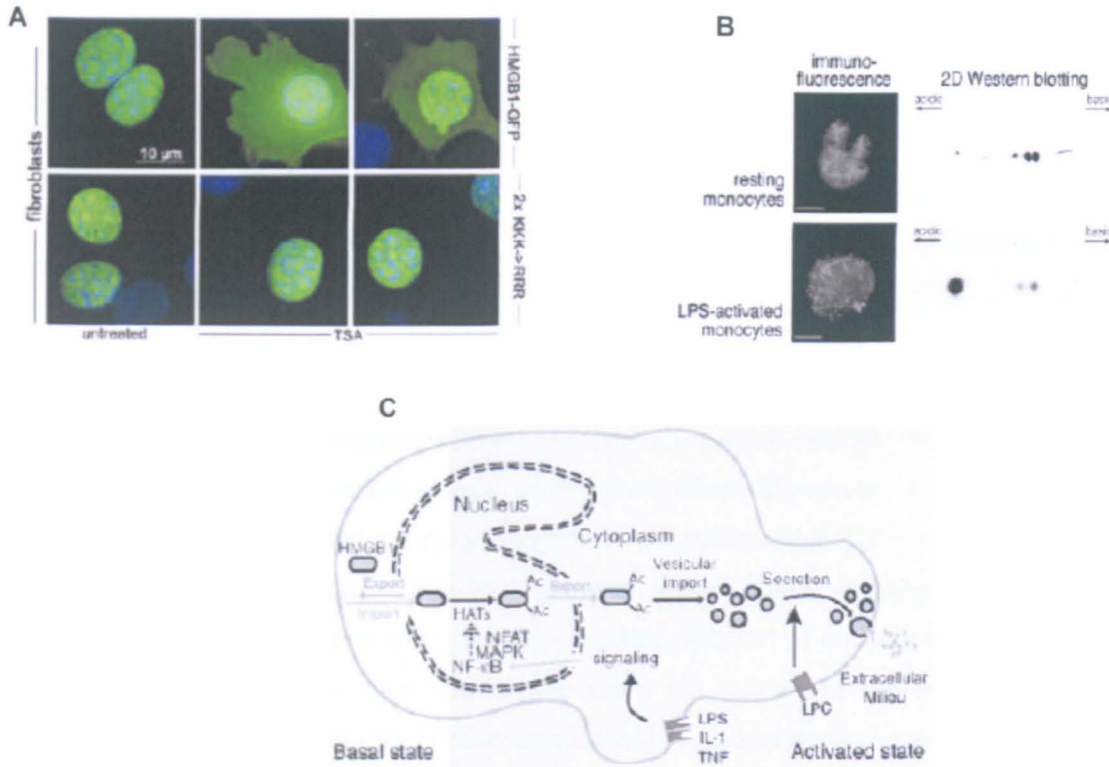
**C. D.** TUNEL staining for apoptotic cells in testis seminiferous tubules of *Hmgb2*<sup>-/-</sup> mice, showing the high content of dead cells.

**E. F.** Haematoxylin-eosin stained sections of testis from a 163-day *Hmgb2*<sup>-/-</sup> mouse. The regular periphery-to-lumen succession of spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa is lost, cells are separated by gaps, and degenerated Sertoli cells with large vacuoles are present.



## HMGB1 nucleus to cytosol translocation and extracellular release

LPS is a trigger that initiates the cellular biochemical modifications that drive HMGB1 secretion. HMGB1 at first exits the nucleus (Bonaldi et al 2003), then enters the secretory vesicles (Gardella et al 2002) and finally reaches the external milieu.



### 7) HMGB1 acetylation and nucleus-cytosol translocation (Bonaldi et al 2003)

**A. HMGB1 moves to the cytoplasm following TSA treatment** Exposure of mouse fibroblasts to 10 ng/ml TSA for 1 h causes a significant relocation of HMGB1-GFP to the cytoplasm; no vesicles are recognizable. The mutation of six lysines to arginines (2XKKK $\rightarrow$ RRR) prevents the cytoplasmic accumulation of HMGB1-GFP, even after TSA treatment.

**B. HMGB1 is multiply acetylated in activated monocytes** LPS-activated human monocytes hyperacetylate HMGB1 and accumulate it in cytoplasmic vesicles. Macrophages purified from peripheral blood were cultured overnight, with or without LPS. HMGB1 is nuclear in unstimulated macrophages, as opposed to nuclear plus vesicular in LPS-activated macrophages. Aliquots of untreated and LPS-activated macrophages were freeze-thawed, and about 400  $\mu$ g of total protein extract was loaded onto 2D gels, blotted and immunodetected with anti-HMGB1. Note the major additional HMGB1 spot in activated macrophages.

**C. The control of HMGB1 secretion in professional inflammatory cells** In all cells, including resting inflammatory cells, HMGB1 shuttles between nucleus and cytoplasm; nuclear import is active, and the protein migrates back to the cytoplasm via passive diffusion and CRM1-mediated active export. When HMGB1 is underacetylated (after TSA treatment), the rate of nuclear import exceeds that of rediffusion plus export, and the protein appears predominantly or solely nuclear. Upon activation of inflammatory cells through binding of IL-1 $\beta$ , TNF- $\alpha$ , LPS or HMGB1 itself to their own receptors, the NF- $\kappa$ B and MAP kinase (MAPK) pathways are activated.

Phosphorylated MAPKs migrate to the nucleus, where directly or via adaptor proteins they activate histone acetylases or inhibit deacetylases. This in turn promotes acetylation of HMGB1. Exported acetyl-HMGB1 cannot return to the nucleus. Myeloid cells are equipped with secretory lysosomes, a variety of lysosomes that can be secreted upon appropriate stimulation and that can accumulate IL-1 $\beta$  or HMGB1, presumably through specific transporters embedded in the lysosomal membrane. Upon binding of LPC (an inflammatory lipid) to its own receptor, the secretory lysosomes carrying HMGB1 fuse with the plasma membrane and secrete their cargo.

The process of HMGB1 exocytosis by macrophages during the inflammatory burst can be subdivided in three fundamental steps:

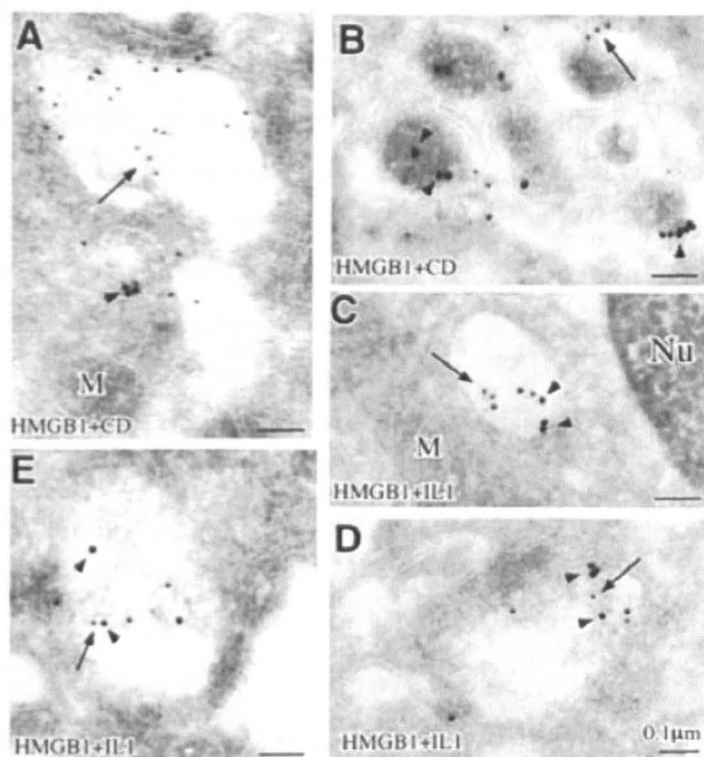
- 1) Translocation of HMGB1 from the nucleus to the cytosol
- 2) Charging of HMGB1 inside cytoplasmic organelles (secretory lysosomes)
- 3) Fusion of containing HMGB1 organelles with the plasma membrane and subsequent release of HMGB1 in the external milieu.

a) The first step was investigated in detail by Bonaldi et al in 2003.

The pattern of 2D-PAGE electrophoretic mobility of modified HMGB1 from macrophagic cells (Figure 7) is compatible with multiple lysine acetylation. Moreover, a deacetylase inhibitor, trichostatin A (TSA), causes the relocalization of a fraction of HMGB1 from the nucleus to the cytoplasm. Mutation of six lysines to glutamine, which mimic acetylated lysine due to the absence of positive charges, also causes the relocalization of a fraction of HMGB1 from the nucleus to the cytoplasm. Mutation of the same six lysines to arginine (which cannot be acetylated) makes HMGB1 localization unresponsive to deacetylase inhibitors (Figure 7).

b) The second exocytosis step is the point under investigation in my work.

HMGB1 utilizes an active transporter to be internalized inside vesicles. Gardella in 2002 found that monocytes/macrophages LPS stimulate release of both, HMGB1 and IL-1 $\beta$  and that these two cytokines partially co-localize inside a secretory-competent lysosomes-type organelle, the “secretory lysosomes”.



**8) IL-1 beta and HMGB1 co-localize with cathepsin D** (Gardella et al 2002) Double immunogold labeling of HMGB1 and cathepsin D (A and B) and HMGB1 and IL-1beta (C and E). Arrows point to small gold particles (HMGB1), and arrowheads point to large gold particles (cathepsin D in A and B, IL-1beta in C and E).

(Figure 8). Secretory lysosomes are a special class of lysosomes, which are present almost exclusively in hemopoietic cells, in keeping with their major role in immune and inflammatory response (Blott and Griffiths 2002).

In spite of its presence inside secretory organelles, HMGB1 lacks a signal peptide that would direct it to the exocytotic pathway via Endoplasmatic Reticulum and Golgi apparatus. The absence of this signal is shared with a small number of other secreted proteins, called leaderless proteins, one of which is IL-1beta.

My thesis work begins at this point. I wanted to understand how HMGB1 can traverse the membrane of secretory lysosomes and accumulate inside them.

c) The third step, lysosome degranulation, needs second signal(s), necessary to trigger the fusion of secretory lysosomes with the cellular membrane.

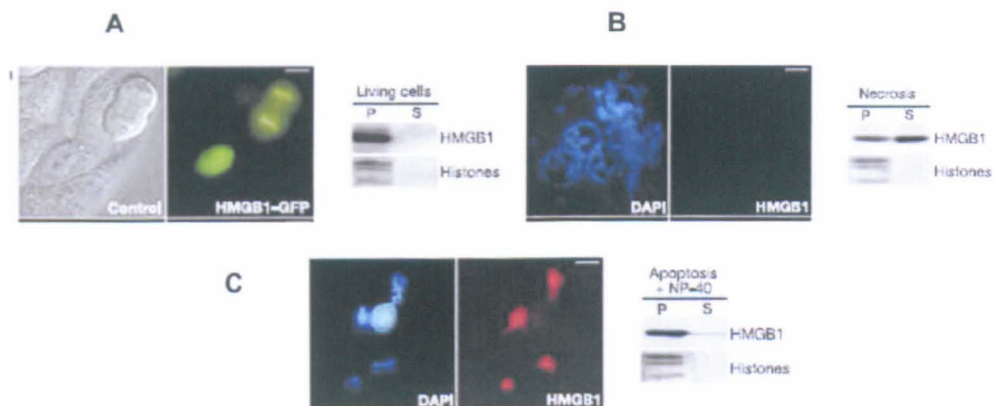
Lysosome degranulation can be stimulated by ATP and lysophosphatidylcholine (LPC), respectively, through activation of two receptors, the purinergic receptor P2X7 and the G-protein coupled receptor G2A (Andrei et al 2004; MacKenzie et al 2001).



### HMGB1 passive release

In 2002 Scaffidi et al demonstrated that HMGB1 passive release, can serve as a diffusible signal of unprogrammed death, which can be used as a cue to nearby cells. Necrotic embryonic fibroblasts from Hmgb1<sup>-/-</sup> mice display a greatly reduced ability to promote inflammation, which indicates that HMGB1 release can signal cellular damage to neighbouring cells. Apoptotic cells do not release HMGB1; the protein remains anchored to chromatin even after secondary necrosis and autolysis, and cannot promote inflammation even if apoptotic cells are not cleared by phagocytic cells (Figure 10). Apoptotic cells are not the result of a present an immediate insult and do not trigger inflammation in physiological conditions. The way in which apoptotic chromatin binds HMGB1 is still unknown.

Core histones, although more abundant than HMGB1, would probably not be good signals of necrosis, as they remain anchored to the insoluble chromatin of necrotic cells.



**10) HMGB1 is associated to the chromatin of living and apoptotic but not necrotic cells** (Scaffidi et al 2002) Both the medium bathing the cells (S) and the cells (P) were analysed by SDS-PAGE. Histones were visualized by Coomassie blue staining, HMGB1 by immunoblotting or immunostaining with antibody to HMGB1, DNA by DAPI.

- A. Living cells expressing HMGB1-GFP, imaged by differential interference contrast and in green fluorescence.
- B. Necrotic cells with no permeabilization. The amount of HMGB1 in the medium was proportional to the number of necrotic cells (about 50%).
- C. Apoptotic cells with permeabilization.

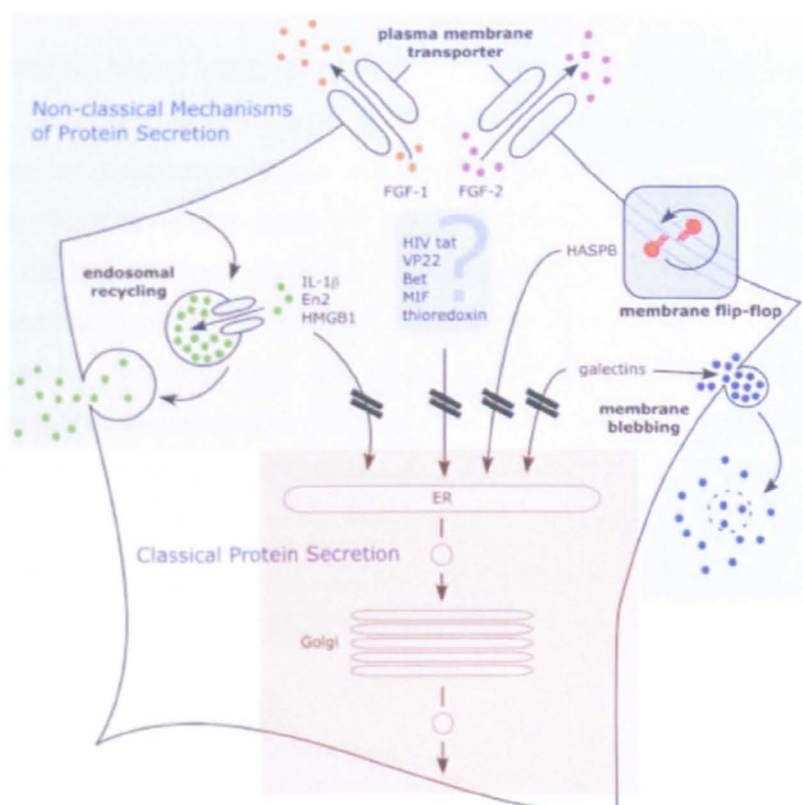
### Leaderless proteins

Secreted soluble proteins typically possess an N-terminal signal (leader sequence) that is able to address the naïve protein inside ER during ribosomal mediated translation. Secreted

proteins follow a vesicular transport via the Golgi apparatus to the cell surface (Figure 11); this exocytosis pathway is known as the classical ER/Golgi dependent secretory pathway (Rubartelli and Sitia 1991).

In the past 10 years a small set of proteins have been identified that are released in the external environment of the cells but that do not possess leader sequences (Figure 11). The leaderless secreted proteins described up to now are:

- CNTF (Ciliary Neurotrophic Factor)
- Coagulation factor XIII, alpha chain
- Beta-galactoside-binding protein
- EMAP II (Endothelial/monocyte activating polypeptide II)
- FGF1 (Fibroblast Growth factor 1)
- FGF2 (Fibroblast Growth factor 2)



**11) Cargo proteins and potential export routes of leaderless and classical protein secretion (Nickel 2005)**

Non classical mechanism: 1) endosomal recycling 2) plasmamembrane transporter 3) flippases/floppases 4)Blebbing.

Classical mechanism: ER/Golgi route.

- Glia activating factor (FGF9)
- HME2 (Homeobox protein engrailed-2)

IL1beta (Interleukin 1 beta)  
 IL18 (Interleukin 18)  
 IL-1RA (IL-1 receptor antagonist)  
 LEG3 (Galectin-3)  
 Mammary-derived growth inhibitor  
 MIF (Macrophage migration inhibitory factor)  
 Prothymosin  
 PD-ECGF (Platelet-derived endothelial cell growth factor)  
 THIO (Thioredoxin)  
 THTR (Thiosulfate sulfurtransferase)  
 Tat (HIV1)  
 ICE (Caspase 1/IL-1 beta converting enzyme)  
 Annexin 1

As reviewed by Nickel 2005, at least four different models of non classical export can exist. IL-1beta, En2 and HMGB1 export involves import into intracellular vesicles, which are probably endosomal subcompartments (secretory lysosomes). FGF-1 and FGF-2 probably reach the extracellular space by direct translocation across the plasma membrane, but they apparently use distinct transport systems. The *Leishmania* cell surface protein HASPB also translocates directly across the plasma membrane and requires that the protein be membrane-anchored through dual acylation at the N-terminus. Therefore, a flip-flop mechanism is required to translocate the protein in the outer layer of the plasma membrane. The final postulated pathway of unconventional protein secretion involves the formation of exosomes, vesicles that form on the outer surface of the cell in a process known as membrane blebbing. Exosomes are labile structures that release their contents into the extracellular space. It has been suggested that this pathway may be used by the galectins (Figure 11).

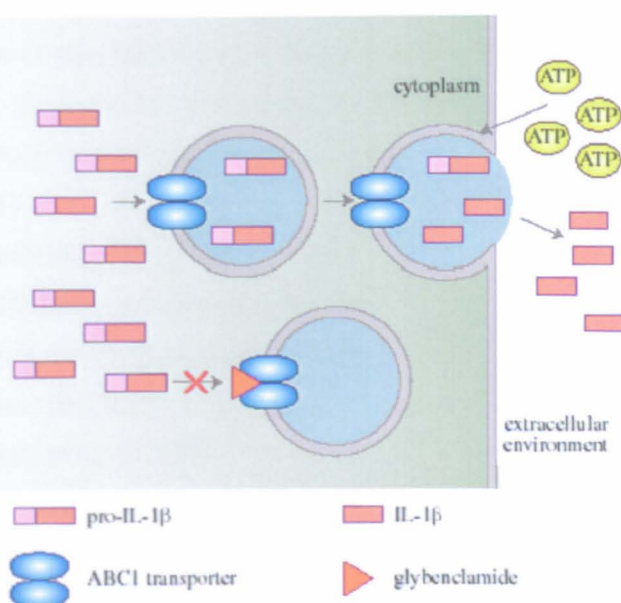
### **Interleukin-1beta**

Interleukin-1beta (IL-1beta) belongs to the Interleukin-1 family, which has three other members: IL-1alpha, IL-1 receptor antagonist and IL-18.

Interleukin-1 proteins are involved in the inflammatory response, and are the endogenous pyrogens which stimulate the release of prostaglandin and collagenase from synovial cells. IL-1 biological activity is extracellular but the protein lacks the secretory signal sequence (Rubartelli et al 1990).

In mammals IL-1 $\beta$  is produced in response to many stimuli which include LPS, numerous microbial products, cytokines (TNF, INF $\gamma$ , GM-CSF and IL-2), T-cell/antigen presenting cell interactions and immune complexes (Stylianou and Saklatvala 1998).

IL-1 $\beta$ , after macrophage stimulation by LPS, is contained inside secretory lysosomes (Hamon et al 1997). IL-1 $\beta$  is synthesized by monocytes/macrophages as a 35 kDa precursor which accumulates in the cytosol (Singer et al 1988). Caspase-1/Interleukin Converting Enzyme (ICE) then processes pro-IL-1 $\beta$  into the mature form of about 17 kDa. ICE is present in the cytosol as a p45 inactive prozyme, the ICE active form is p10/p20 (Figure 12) (Singer et al 1995; Ayala et al 1994). IL-1 $\beta$  maturation takes place in the secretory lysosomes (Andrei et al 1999) where both IL-1 $\beta$  and active ICE co-localize. A general ABC transporters inhibitor, glybenclamide, inhibits IL-1 $\beta$  vesicle loading (Hamon et al 1997), possibly by inhibiting the transporter ABCA1.



**12) IL-1 $\beta$  exocytosis model** Pro-IL-1 $\beta$  is present in the cytosol and is loaded inside the secretory lysosomes by the activity of a glybenclamide sensitive ABC transporter (ABCA1).

### ABC transporters superfamily

The ATP-Binding-Cassette (ABC) transporter superfamily is composed of a set of transmembrane proteins that translocate compounds across plasma and intracellular

membranes. The name ABC transporters was first introduced by Higgins (1992), who cloned and sequenced the first ABC transporter, the histidine permease (Higgins et al 1982).

All human and mouse ABC genes have standard nomenclature, developed by the Human Genome Organization (HUGO). Details of the nomenclature scheme can be found at: <http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html>.

In summary the ABC transporter superfamily is organized in seven sub-branches:

- 1) Subfamily A (ABCA) - ABC1
- 2) Subfamily B (ABCB) – MDR/TAP
- 3) Subfamily C (ABCC) – CFTR/MRP
- 4) Subfamily D (ABCD) – ALD
- 5) Subfamily E (ABCE) – OABP
- 6) Subfamily F (ABCF) – GCN20
- 7) Subfamily G (ABCG) – WHITE

Mutations in some of the ABC transporter genes are the cause of a variety of human diseases with Mendelian or complex inheritance; in fact, many of these genes were originally identified by positional cloning of human disease genes:

Tangier disease (ABCA1)

Immune deficiency (ABCB2-3/TAP1-2)

Progressive familial intrahepatic cholestasis (ABCB4)

X-linked sideroblastic anemia and ataxia (ABCB7)

Dublin-Johnson syndrome (ABCC2)

Pseudoxantoma elasticum (ABCC6)

Cystic Fibrosis (ABCC7/CFTR),

Familial persistent hyperinsulinemic hypoglycemia of infancy (ABCC8)  
adrenoleukodystrophy (ABCD1)

Sitosterolemia (ABCG5/8)

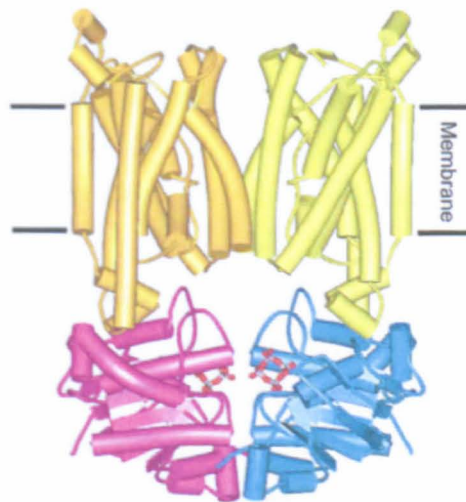
ABC transporters perform translocation of different toxic compounds, drugs, ions, peptides, lipids or bile salts, across the hydrophobic bilayer of the plasma membrane, often against concentration gradients. The translocation process consumes energy that is obtained from ATP hydrolysis.



### ABC transporter structure

ABC transporters are typically composed of 4 domains (as depicted in figure 13, Higgins and Linton 2004), two transmembrane domains (TMD) formed by several hydrophobic alpha-helices and two Nucleotide Binding Folds (NBF). The four domains can be encoded by two different polypeptides which can dimerise, or can be fused into a single protein.

The ABC transporters were classified on the basis of the sequence of their ATP-binding domains, the NBF. The NBF displays a typical and conserved structure: it is composed of two Walker domains, A and B, separated by the C signature.



**13) Typical ABC transporter structure (*E. coli* BtuCD homologue of mammalian ABC transporter)** ( Higgins and Linton 2004) In yellow and orange TMDs, in purple and cyan NBFs. TMDs are composed by several hydrophobic alpha helices spanning the membrane. NBFs are alpha/beta Rossmann folds.

ABC transporters are large membrane proteins and for this reason it is difficult to crystallize them in order to determine their structure and the conformational changes occurring during substrate translocation.

Chang and Roth in 2001 described the X-ray structure to a resolution of 4.5 angstroms of the *E. coli* homolog, MsbA, of the multiple drug resistance transporter (MDR1). MsbA is a lipid flippase that has a cone-like shape, organized as a homodimer with each subunit containing 6 transmembrane alpha-helices and a nucleotide-binding domain.

Higgins and co-workers in 1997 described the shape of MDR1 (purified from Chinese hamster cells) using electron micrograph image analysis. The transporter looks like a cylinder with a diameter of 10 nm and a height of 8nm, it also has a central cavity with a diameter of 5 nm (Rosenberg et al 1997).

Up to now, two possible models exist to explain the mechanism by which ABC transporters perform the translocation:

Flip-Flop (Rayes and Chang 2005)

ATP switch model (Higgins and Linton 2004).

### **Drug resistance and MDR/MRP**

In 1976 Juliano and Ling discovered a large glycoprotein in the plasma membrane of multiple drug resistant cells, the MDR1/ABCB1, and they considered it a primary active drug pump able to confer detoxification capacity to the cells that overexpress it.

In 1992 Cole and coworkers defined MRP (now MRP1/ABCC1) as a second type of drug pump in multiple drug resistant cells. MRP1 was identified in a multidrug-resistant sub-clone of a small cell lung carcinoma cell line (NCI-H69) that does not overexpress MDR/ABCB1 (Cole et al 1992; Roninson et al 1986; Shen et al 1986; Borst et al 2000; Borst and Efferink 2002).

In 1994 Leier demonstrated that MRP1/ABCC1 has a active GS-X pump activity (Glutathione conjugated compounds pump). The GS-X pump activity was studied in the 80s for its involvement in the Phase III elimination of conjugated organic anions produced by Phase I and Phase II detoxification metabolism of many endo- and xenobiotics; eliminated compounds are mostly glutathione, glucuronide or sulfate conjugated compounds (Ishikawa et al 1994).

A MRP1 substrate is LTC<sub>4</sub>, which derives from arachidonic acid in a series of reactions that at the end culminate in glutathione conjugation (Ishikawa et al 1990).

Definite proof that MRP1 can be the cause of multiple drug resistance came from transfection studies. HeLa cells transfected with MRP1 cDNA displayed a typical multidrug resistance phenotype, accompanied with a reduced intracellular drug accumulation. Similar behavior was found using other cell lines in different and independent laboratories (Cole et al 1994; Grant et al 1994; Hipfner et al 1999).

Newly synthesized MRP1 polypeptide migrates at approximately 170 kDa in SDS-PAGE, consistent with a molecular mass of about 171 kDa predicted from its cDNA sequence. The immature form of the transporter is rapidly N-linked glycosylated, and the protein acquires a molecular mass of about 190 kDa (Almquist et al 1995).

### **ABC transporters involved in drug resistance and peptide transport**

The ABCC/MRP sub-family is composed of twelve members and the ABCB/MDR sub-family of eleven members in humans.

These proteins have been identified as active, ATP-dependent membrane transporters for various drugs, organic anions with anticancer activity and also peptides. MDR1/PGY1/ABCB1 and MRP1-5/ABCC1-5 lead to multidrug resistance in tumor cells (Dean and Annilo 2005).

Remarkably, in 2001 it was demonstrated that MRP1/ABCC1 is able to translocate peptides across the plasma membrane (de Jong et al 2001). MDR1/PGY1/ABCB1, a member of the ABCB sub-family, was shown to transport a toxic peptide (ALLN) and other synthetic peptides across the cellular membrane (Sharma et al 1992; Lee et al 1999; Sarkadi et al 1994).

Mostly peptide transport capacity has been described for some ABCB transporters sub-family members, TAP1/ABCB2 (Antigen Peptide Transporter 1) and TAP2/ABCB3 (Antigen Peptide Transporter 2). TAP proteins are responsible for the translocation of antigenic peptides from the cytosol to the ER compartment of class I MHC expressing cells. MHC I molecules are synthesized in the ER and then exposed onto the cellular membrane already bound to the antigenic peptides. The membrane separating cytosol and the internal lumen of the ER is peptide impermeable, for this reason a pore or a transport molecule consuming energy is necessary to let the antigen enter the lumen. TAP proteins function as dimers composed of two subunits, TAP1 and TAP2, of about 75 kDa, which contain a NBF each. They are localized in the ER and cis-Golgi apparatus even if they do not possess the ER retention signal. TAP proteins are organized in a head-head/tail-tail orientation and the hetero-dimer forms a peptide binding pocket on the cytosolic side of the membrane in which TAP proteins are imbedded (Vos et al 1999; 2000).

In 2001 Young found that the *Saccharomyces cerevisiae* ABC transporter Mdl1 is involved in peptide export from mitochondria. Mdl1 is homologous to human TAP proteins and belongs to the ABCB sub-branch.

Moreover, ABCA1/CERP, a component of the ABCA sub-family, was suggested to be involved in the exocytosis of two leaderless proteins, IL-1beta and MIF (macrophages Migration Inhibitory Factor). These translocation studies were performed mainly using a pharmacological approach without giving a real and full demonstration of the molecular mechanism and an explanation of the translocation phenomenon. In this context Probenecid is reported as a specific drug inhibitor of the ABCC sub-family members by several people; Flieger in contrast observed a Probenecid inhibitory effect on ABCA1 transporter (Wein et al 2004, Ferreira et al 2005a, Ferreira et al 2005b, Marty et al 2005, Hamilton et al 1993; Flieger et al 2003; Hamon et al 1997; Rubartelli et al 1990; Webster et al 2002; Michot et al 2006; Lucia et al 2005; Jorajuria et al 2004; Olson et al 2002).



A possible explanation of this conflict is that the cellular system that Flieger utilized is representative only of the behaviour of monocyte/pro-macrophages, but not of the fully differentiated macrophages. Fully differentiated macrophages, however, are the main target of my studies and more specifically of the IL-1 $\beta$  exocytosis inhibition experiments.

### **Mrp1<sup>-/-</sup> mouse phenotype**

Mrp1<sup>-/-</sup> mice have defects in the innate and adaptive immune response (Wijnholds et al 1997). In 2000 Robbani et al showed that Mrp1<sup>-/-</sup> mice have impaired dendritic cell (DC) migration from skin to lymph nodes, and that the DC migratory ability can be partially restored by LTC<sub>4</sub> administration. It was also shown that MK571, an antagonist of MRP1, inhibits emigration of DCs from skin explants, and this suggested that MRP1 regulates DC migration by transporting LTC<sub>4</sub>.

In 2001 Schultz et al showed that Mrp1<sup>-/-</sup> mice intranasally inoculated with *Streptococcus pneumoniae* were resistant to pneumonia. The mice displayed reduced inflammation marker levels in the lungs (TNF, IL-6, INF $\gamma$ ) and diminished mortality, if compared with mice inoculated with saline. The author also described a block in the extracellular release of LTC<sub>4</sub>.

In 2002 Verbon et al analyzed the T-helper 1 immune response in Mrp1<sup>-/-</sup> mice after intranasal inoculation of *Mycobacterium tuberculosis*. Two weeks after infection the mice had reduced levels of INF $\gamma$ , and reduced granuloma formation in lungs.

The inflammation resistant phenotype of Mrp1<sup>-/-</sup> mice has been interpreted mainly as a consequence of impediment in the release of LTC<sub>4</sub>.

### **Unfolding and transmembrane translocation**

Proteins have to fold into well-defined three dimensional structures to function correctly, but unfolding is also essential for several processes in the cell. Protein unfolding is a crucial step for importing some proteins into mitochondria or chloroplasts and in degradation of regulatory proteins by ATP-dependent proteases (Eilers and Schatz 1986; Rassow et al 1990; Walker et al 1996). The necessity of unfolding was demonstrated during ER import (Paunola et al 1998) and lysosomal import (Salvador et al 2000).

At the experimental level key findings have been made using mitochondrial reporter proteins and FGF-2, which were fused with dihydrofolate reductase (DHFR), an enzyme whose three-dimensional structure can be stabilized by the folate derivative, methotrexate (Eilers and Schatz, 1986; Wienhues et al., 1991; Backhaus et al 2004). Employing the DHFR domain as part of a specific reporter molecule, protein translocation across a membrane can

be inhibited in the presence of methotrexate and for sure this kind of experiment is only an indirect proof of unfolding necessity for the whole reporter chimerical protein.

In my work I utilize the chimera between HMGB1 and DHFR and methotrexate treatment to indirectly demonstrate that HMGB1 exocytosis unfolding is needed. Is reasonable that HMGB1 must unfold because of MRP1 tight pore and gate, even if, as I seed before, this experiment is only unindirect explanation of the translocation mechanism, but it seems reasonable to say that HMGB1 can not go thought MRP1 without modifying its structure for steric reasons.

## Materials and Methods

### Chemicals and antibodies

Glybenclamide (Sigma G0639), Ethacrynic Acid (Sigma E4754), 4,4' diisothiocyanatosilbene-2,2'- disulfonic acid disodium salt (DIDS) (Sigma D3514), Probenecid (Sigma P8761), Verapamil (Sigma V4629), Lipopolysaccharide (LPS) (Sigma L2654), Phorbol 12-myristate 13-acetate (PMA) (Sigma P8139), LPS binding protein (LBP) (HBT HC4010), Lysophosphatidylcholine (LPC) (Sigma L4129), Adenosin-5'-triphosphate disodium salt (ATP) (Boehringer 519979, dissolved in H<sub>2</sub>O pH 7.2, 20mM), L-buthionine-sulfoximine (BSO) (Sigma B2515), Methotrexate (Sigma M8407), MK571 (Alexis), reduced glutathione (GSH) (Sigma G1404), oxidised glutathione (GSSG) (Sigma G4376), glutathione reductase (EC1.6.4.2) (Sigma G3664), NADPH (Sigma N1630), N-Ethylmaleimide (Sigma E1271), Hydrogen Peroxide- H<sub>2</sub>O<sub>2</sub> (Sigma U8879)

Monoclonal antibody anti-IL-1beta (Cell Signaling 2022), monoclonal antibody anti-MRP1 (human QCRL-3) (Alexis 801011L001), monoclonal mouse anti-MRP1 (Chemicon MAB4147), polyclonal antibody anti-HMGB1 (BD 556528), monoclonal antibody anti-CD14 (BD 555396), monoclonal antibody anti-beta-actin (Sigma A5441), monoclonal antibody against glutathione bound to protein (PSSG) (ViroGen 101-A), polyclonal antibody anti-LDH (Chemicon AB1222),

### *E. coli* strains

**DH5 $\alpha$ :** Strain with genotype *supE44 DlacU169 (F80 lacZDM15) hsdR17 recA1 end A1 gyrA96 thi-1 relA1*. A recombinant-deficient suppressing strain used for plating and growth of plasmids.

**BL21(-):** Strain with genotype *hsdS gal ( $\lambda$  clts857ind 1 Sam7 nin5 lacUV5-T7 gene1)*. A strain used to express at high levels genes under the control of T7 promoter. It lacks the plasmid pLysE, coding for T7 phage lysozyme which inhibits the RNA polymerase basal activity.

*E. coli* bacteria were generally grown in LB (Luria-Bertani) Medium, supplemented with selective antibiotics.

LB (per liter) bacto-tryptone 10 g ; bacto-yeast extract 5 g; NaCl 10

### Primary cells and cell lines

Primary mouse macrophages were isolated by peritoneal washing. Mice were injected with 3 ml of a 3% thioglycollate solution in the peritoneal cavity, After 2 or 3 days, mice were ether

anesthetized, and injected intraperitoneally with 5 ml PBS (Gibco); after 10 min the fluid was recovered. Macrophages were incubated in RPMI 1640 (Gibco) complete medium plus 20  $\mu$ M beta-mercapthoethanol for 5 hours immediately after extraction from the peritoneum. They were then cultured in RPMI 1640 complete medium supplemented with 10% fetal calf serum (FCS-Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100  $\mu$ g/ml streptomycin (Gibco).

The human pro-monocytic THP-1 cell line, established from a diffuse histocytic lymphoma, is an *in vitro* model of monocyte to macrophage differentiation. THP-1 cells were maintained in complete RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, or OptiMem (Gibco), and differentiated into macrophages by treating with 200 nM PMA for 3 to 48 hours. Stimulation with LPS (5  $\mu$ g/ml) was performed 18 to 48 hours after the end of differentiation. THP-1 cell line was a Massimo Alessio kind gift.

Human ovarian carcinoma 2008 cells, and stable transfectants thereof –2008(MRP1), 2008(MRP2), 2008(MRP3) and 2008(MRP5)– were a kind gift from Piet Borst. These cells, HeLa cells and stable transfectants from thereof –HeLa(sCD14), HeLa(MRP1), HeLa(MRP2), HeLa(MRP3), HeLa(MRP5), HeLa(MDR3) and HeLa(CFTR)– were cultured in complete DMEM (Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Gibco), or OptiMem (Gibco).

HeLa cell line were cultured in complete DMEM (Gibco) supplemented with 10% fetal calf serum (FCS- Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Gibco), or OptiMem (Gibco). HeLa cell line was available in Marco Bianchi's laboratory.

C1 cell line (Hmgb1-/- mouse fibroblasts) were cultured in complete DMEM (Gibco) supplemented with 10% fetal calf serum (FCS- Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Gibco), or OptiMem (Gibco). C1 cell line was available in Marco Bianchi's laboratory and was generated by Paola Scaffidi.

### Protein secretion

One million THP-1 cells were plated in 3 cm wells, and differentiated into macrophage as described above. One day after PMA induced differentiation, cells were stimulated overnight with 5  $\mu$ g/ml LPS in OptiMem (Gibco) medium (1 ml) in the presence or absence of various inhibitors (glybenclamide 250  $\mu$ M, DIDS 150  $\mu$ M, ETA 50  $\mu$ M, Probencid 2 mM, Verapamil 100  $\mu$ M and MK571 50-300  $\mu$ M. Degranulation of secretory lysosomes was activated adding 0.5  $\mu$ M ATP 15 min before supernatant collection. Proteins in cell supernatants were

precipitated in 50% acetone, and resolved by reducing SDS-PAGE. HMGB1, IL-1 $\beta$  were detected by western blotting.

### **TLR4 activation**

The HeLa(sCD14) stable transfectant releases sCD14 in the supernatant. Medium conditioned by HeLa(sCD14) cells was collected, filtered and supplemented with 2  $\mu$ g/ml LPS and 100 ng/ml LBP. The medium containing LPS/LBP/sCD14 was used to activate TLR4 HeLa (or 2008) derived cell lines.

### **Soluble CD14 (sCD14) cloning**

sCD14 cDNA sequence was amplified by Total RNA was extracted with TRIzol (Invitrogen) from PMA-differentiated THP-1 cells, and RT-PCR was performed using the primers sCD14Fw gtc cgg gat ccc cac cat gga gcg cgc gct ctg c and sCD14Rev tcc agg aat tct tac agc acc agg att ccc ga). The RT-PCR product was then cloned BamHI/EcoRI in the pcDNA-3 mammalian expression vector. The construct was transfected in HeLa cells using FuGENE6 (Roche), G418-resistant clones were isolated and tested for sCD14 production by immunofluorescence.

### **ABC transporter cloning**

MRP1 cDNA was amplified by PCR from pj3omega-MRP1 (X\_78338), a gift from P. Borst, using the primers MRP1Fw aaa gat atc atg gcg ctc cgg ggc ttc and MRP1Rev aaa gcg gcc gc tac aac caa ttc ctc, and then cloned in pcDNA (EcoRV/NotI).

MRP2 cDNA was amplified by PCR from pGEM3-MRP2 (U\_49248), a gift from P. Borst, using the primers MRP2Fw aaa aag ctt atg ctg gag aag ttc tgc and MRP2Rev gaa ttt tgt gct gtt cac att, and then cloned in pcDNA (HindIII).

MRP3 cDNA was amplified by PCR from pGEM7-MRP3 (AF\_009670), a gift from P. Borst, using the primers MRP3Fw aaa gaa ttc atg gac gcc ctg tgc ggc and MRP3Rev aaa ctc gag ctc atc agc ttg atg cgc, and then cloned in pcDNA (EcoRI/XhoI).

MRP5 cDNA was amplified by PCR from pGEM5-MRP5 (NM\_005688), a gift from P. Borst, using the primers MRP5Fw aaa aag ctt atg aag gat atc gac ata gga and MRP5Rev aaa gaa ttc gcc ctt gac agc gac ctt, and then cloned in pcDNA (HindIII/EcoRI).

CFTR cDNA was amplified by PCR from pBS-CFTR (NM\_000492), a gift from M. Conese, using the primers CFTRFw.: aaa ggt acc atgcag agg tcg cct ctg and CFTRRev aaa ctc gag aag cct tgt atc ttg cac, and then cloned in pcDNA (KpnI/XhoI).

MDR3 cDNA was amplified by PCR from pj3omega-MDR3 (M\_23234) from ATCC using the primers MDR3Fw aaa aag ctt atg gat ctt gag gcg gca aag and MDR3Rev aaa ccc ggg taa gtt ctg tgt ccc agc, and then cloned in pcDNA (HindIII/EcoRV).

The plasmids were transfected in HeLa cells using FuGENE6 (Roche) following manufacturer's protocol. ABC transporter overexpressing cell clones were isolated by limiting dilution approach after G418 (400µg/ml) selection, thanks to the neomycin resistance given by the transfected plasmidic construct. Each cell clone was analyzed by immunofluorescence (as described elsewhere in the thesis) using antibodies addressed against the different ABC transporters, in order to test for transporter plasmamembrane localization, and level of overexpression (untransfected cells as negative control). The overexpressed ABC transporters were considered functional once were found expressed on the cell plasmamembrane even if formal proof was not provided.

### **Immunofluorescence**

Primary macrophages were plated on acid-washed glass coverslips and maintained in the appropriate culture medium and experimental conditions. Immunofluorescence and imaging was described in Bonaldi et al (2003). In brief, cells were fixed in PHEM buffer (36.8 g/l PIPES, 13 g/l HEPES, 7.6 g/l EGTA, 1.99 g/l MgSO<sub>4</sub>, titrated to pH 7.0 with KOH) plus 3.7% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were then treated for 5 minutes with HEPES-based permeabilization buffer (300 mM sucrose, 0.2% Triton X-100) and then for 15 minutes with Blocking buffer (3% Bovine Serum Albumin in PBS). Primary and secondary fluorophore conjugated antibodies were diluted in PBS+BSA 0.2%. Hoechst 33342 (1.5 µg/ml) in PBS+BSA 0.2% was used as counterstaining.

Cells expressing GFP fusion proteins were PFA-fixed, Hoechst 33342 stained and imaged.

Cells were imaged using an Olympus 100x or 60x/1.4NA Plan Apo oil immersion objective lens on a DeltaVision Restoration Microscopy System (Applied Precision, Issaquah, WA, USA) built around an Olympus IX70 microscope equipped with mercury-arc illumination. Filters were from Chroma Technology Corp. (Brimley, VT, USA): Hoechst 33342 excitation 360/40, emission 457/50; GFP excitation 490/20, emission 528/38. Twenty optical sections spaced by 0.5 µm were collected with a Coolsnap\_Hq/ICX285 CCD camera (Photometrix, Tucson, AZ, USA) and deconvolved by the constrained iterative algorithm available in the SoftWoRx 2.50 package (Applied Precision) using 10 iterations and standard parameters. Each image measured 512x512 pixels, and effective pixel size was 0.106 µm.

### **Immunoprecipitation**

THP-1 cells, PMA differentiated and LPS stimulated, were lysed in JS buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Glycerol, 1% Triton-X100, 1.5 mM MgCl<sub>2</sub>); the protein concentration was determined by Bradford protein assay. Whole cells lysate (1 mg of in 500 µl final volume) was incubated with Dynabeads protein G (2 µg, equal to 34 million beads) (Oxoid) for 1 hour at 4°C. Beads were washed using JS buffer before loading.

### **Radiolabelling of the GSH/GSSG intracellular pool.**

Radiolabelling and protein synthesis block was performed as described in Fratelli et al (2003; 2002). Experiments were performed in presence or absence of BSO 20 µM. HMGB1 was immunoprecipitated from THP-1 cell lysate (after PMA differentiation and LPS stimulation) avoiding all reducing agents. In brief, macrophages derived from THP-1 cells were pre-incubated for 1 hour in HBSS (Hanks' balanced salt solution, Gibco) with 50 µg/ml cycloheximide and then stimulated with 5 µg/ml LPS in the presence of 8 mCi/ml L-[<sup>35</sup>S]-Cys (specific activity 1000 Ci/mmol). Cells were lysed using JS buffer plus 50 mM N-ethylmaleimide, MRP1 and HMGB1 were immunoprecipitated as described above, and samples were resolved by non-reducing SDS-PAGE. Radioactivity incorporation was detected by autoradiography of the dried gel.

### **HMGB1-DHFR-GFP chimeric protein and unfolding assay**

Human DHFR cDNA (NM\_000791) was amplified by RT-PCR from total RNA extracted from HeLa cells using the primers DHFR<sub>FW</sub> ata aag ctt atg gtt ggt tgg cta aac tgc and DHFR<sub>REV</sub> ata acc ggt aaa tca ttc ttc tca tat act. Rat HMGB1 cDNA (NM\_012963) was amplified by PCR from pEGFP-HMGB1 (Scaffidi et al 2002) using the primers B1<sub>FW</sub>: ata ctc gag atg ggc aaa gga gat cct aag and B1<sub>REV</sub> ata aag ctt ttc atc atc atc atc ttc ttc; the PCR product was cloned in pEGFP-N1 (XhoI/HindIII) and DHFR orf was cloned in frame with HMGB1 (HindIII/AgeI).

HeLa(MRP1) cells were transiently transfected with pEGFP-N1/HMGB1 (Scaffidi et al 2002) and pEGFP-N1/HMGB1/DHFR. One day after transfection, the cell medium was changed with HeLa(sCD14)-conditioned OptiMem supplemented with LPS (2 µg/ml) and LBP (100 ng/ml) to activate TLR4, in the presence or absence of 20 µM methotrexate (MTX). The day after, cell supernatants were precipitated with 50% acetone and western blotting with anti-GFP was performed.

### Cysteine to Serine HMGB1 mutants

Single and triple cysteine-to-serine HMGB1 mutants (C22S, C44S, C105S and C22/44/105S) were generated by site directed mutagenesis using Pfu DNA polymerase (Promega), pEGFP-HMGB1 (Scaffidi et al 2002) as template and the following primers:

- |                    |                                     |
|--------------------|-------------------------------------|
| 1) HMGB1_Cys_Fw1:  | aaa ctc gag atg ggc aaa gga gat     |
| 2) HMGB1_Cys_Rev1: | ccg act agt ttg cac aaa aaa tgc ata |
| 3) HMGB1_Cys_Fw2:  | gtg caa act agt cgg gag gag cat aag |
| 4) HMGB1_Cys_Rev2: | tga act ctt ctt aga aaa ctc tga gaa |
| 5) HMGB1_Cys_Fw3:  | tct aag aag agt tca gag agg tgg aag |
| 6) HMGB1_Cys_Rev3: | aga act gaa gag gaa gaa ggc cga agg |
| 7) HMGB1_Cys_Fw4:  | ttc ctc ttc agt tct gag tat cgc cca |
| 8) HMGB1_Cys_Rev4: | ttt gaa ttc ttt cat cat cat cat ttc |

Primers 2 and 3 mutagenise cysteine 22, primers 4 and 5 mutagenise cysteine 44, and primers 6 and 7 mutagenise cysteine 105. Primers 1 and 8 amplify all HMGB1 orf adding XhoI and EcoRI restriction sites. Mutant HMGB1 PCR products were cloned in pEGFP-N1 and pcDNA eukaryotic expression vectors. After HMGB1 mutant cloning all the vectors were sequenced by Primm s.r.l. (Milano, Italy) after a restriction analysis was performed. The plasmids were transfected in HeLa or C1 cells using FuGENE6 (Roche) following manufacturer's protocol (G418 selection was not applied) and protein expression was tested by fluorescence microscopy (GFP tagged) or whole cell lysates western blotting analysis against HMGB1.

### Real time-PCR Analysis

The analysis was performed to test MRP1 (NM\_008576.2) MRP2 (NM\_013806.1) and MRP3 (NM\_029600.2) mRNA expression level in mouse primary macrophages.

Total RNA was extracted from primary macrophages (peritoneal wash of Mrp1<sup>-/-</sup> and wt mice) using TRIzol (Invitrogen) and according to the manufacturer's instructions. Total RNA was quantitated by spectrometry and A260/A280 ratios were determined. The mRNA level of a housekeeping gene, GAPDH (NG\_005233.1), was used to normalize different experiments and different total RNA samples, testing serial dilutions of the total RNA; amounts ranging from 5 to 500 ng (linear regression analysis).



2 µg of total RNA were treated with DNase I/RNAase free (Promega) and reverse transcribed using Superscript II Rnase H reverse transcriptase (Life Technologies) and oligo(dT)16 as a template primer (as manufacturer described).

First cDNA stand was diluted (1:500) and quantified by LightCycler instrument (Roche) and LightCycler fast Start DNA master(plus) SYBR green Kit (Roche); procedure was performed as described by the manufacturer.

To quantify MRP1, 2 and 3 mRNA levels, the number of MRPx mRNA copies in WT and KO samples were divided by the amount of total RNA (in nanograms) determined by the number of GAPDH mRNA copies in the same sample. MRPx mRNA levels in these experiments were expressed as the mean copy number of MRPx mRNA molecules in 15 ng of total RNA (each quantification was performed in triplicate).

The primers used were the following:

Mouse MRP1	Fw1.: gta gag ttc cgg gat tac Rev1.: cgc agg ttg tgc agg cgg at
Mouse MRP2	Fw2.: tgg ctg aga tgc gag ag Rev2.: ttg gtc ctt tca cta ctt c
Mouse MRP3	Fw3.: cgc tct cag ctc acc atc at Rev3.: ggt cat ccg tct cca agt ca
Mouse GAPDH	Fwg.: gaa agc tgt ggc gtg atg Revg.: tga ata cgg cta cag caa ca

### **Recombinant HMGB1 glutathionylation *in vitro***

Redox reactions were performed in a 1.5 ml Eppendorf tube in 50 µl reaction volume (buffer: 0,2 mM NaP pH 7.5, 2 mM NaCl, protease inhibitor (Complete-Roche)) for 5 minutes at room temperature. Reagent concentrations were: 20 µg/ml rHMGB1 (714 nM), 4 µM GSH, 4 µM GSSG: 4 µM H<sub>2</sub>O<sub>2</sub>, 4 µM NADPH, 60 U/ml glutathione reductase. Samples were resolved by non-reducing 12% SDS-PAGE. Gels were stained with Coomassie or analysed by western blotting with anti-HMGB1.

Glutathione reductase is a flavoenzyme which utilises NADPH as electron donor. One enzyme unit is defined as the quantity of protein that reduces 1 µmole of GSSG to GSH in 1 min at pH 7.6 and 25°C.

### **Recombinant HMGB1 expression and purification**

The plasmid pT7-7-rHMG1 used for expression of recombinant full-length HMGB1 in bacteria was a kind gift of prof. J.O. Thomas (Cambridge University, UK).

rHMGB1 was produced in the BL21(-) *E. coli* strain, following the protocol of Studier and Moffatt (1986), but with some modifications: briefly, M9 medium was used instead LB (per liter) bacto-tryptone 10 g ; bacto-yeast extract 5 g; NaCl 10 g.

5 X M9 salts (per liter):

Na <sub>2</sub> HPO <sub>4</sub> · 7H <sub>2</sub> O	64 g
KH <sub>2</sub> PO <sub>4</sub>	15 g
NaCl	2.5 g
NH <sub>4</sub> Cl	5 g

M9 (per liter):

5 X M9 salts	200 ml
1 M MgSO <sub>4</sub>	2 ml
1 M CaCl <sub>2</sub>	0.1%

completed with:

- Cas-aminoacids 20 g/L
- Glycerol 0.5%
- Yeast Extract 5g/L
- Glucose 0.4%
- Chloramphenicol 100 µg/ ml

BL21 (-) *E. Coli* transformed with pT7-7-rHMG1 were pre-inoculated in 300 ml medium o/n, then inoculated in 3 L medium (37°C, shaking 200 rpm). IPTG (0.5 mM) was added to the culture when the O.D.=0.7 (RT, shaking 100 rpm). Bacteria were left to grow for 16 hours, then they were collected by centrifugation. Bacteria were resuspended in Buffer L2 (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), using sonication to clarify. NaCl was added to a final concentration 0.5 M and the extract was centrifuged to pellet the debris.

The first purification step is a differential precipitation obtained by adding (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 60% solubility: HMGB1 remains in solution. After 30 minutes on ice, the extract was centrifuged at 10 000 rpm (Heraeus centrifuge model Sepatech GmgH) for 30 minutes. The supernatant was collected, filtered (20 µm) and loaded on a Phenyl-Sepharose Column (Amersham Pharmacia), connected to a FPLC System. rHMGB1 is eluted with stepwise decreasing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation (60%- 50%- 40%- 30%) generated by mixing the following buffers:

buffer A	20 mM HEPES pH 7.9	buffer B	20 mM HEPES pH 7.9
	0.5 mM DTT		0.5 mM DTT
	0.2 mM PMSF		0.2 mM PMSF
	0.2 mM EDTA pH 8.0		0.2 mM EDTA pH 8.0
	0% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		60% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>

Positive fractions were dialysed overnight in buffer A' (see below) and loaded on a Hi-Trap – SP column (Amersham Pharmacia) connected to a FPLC System (Amersham Pharmacia). rHMGB1 was eluted in an increasing gradient of NaCl, mixing the following buffers:

buffer A'	50 mM Hepes pH 7.9	buffer B'	50 mM HEPES pH 7.9
	0.5 mM DTT		0.5 mM DTT
	0.2 mM PMSF		0.2 mM PMSF
	20 mM NaCl		1 M NaCl

rHMGB1 was concentrated in Centricon Cartridges (Millipore) and desalted in final Buffer G; the final concentration was evaluated both by Bradford Method and by Coomassie Staining on 12% SDS-PAGE.

Buffer G:

10 mM NaP pH 7.5  
100 mM NaCl  
0.5 mM DTT  
mM PMSF.

## 2D gel electrophoresis

About 50 µg of purified HMGB1 or 250-400 µg of protein from total extracts were added to 350 µl rehydration buffer (8 M urea, 2% CHAPS, 20 mM dithioerythritol, 0.8% IPG buffer, carrier ampholytes pH 3-10 Non Linear or pH 4-7 Linear). Samples were applied onto ceramic strip holders (Pharmacia Biotech) connecting two electrodes, in contact with 18 cm polyacrylamide gel strips (pH range: 3-10 not linear, or pH 4-7 linear). Isoelectrofocusing (IEF) was performed on IPGphor (Pharmacia Biotech) with 2 different protocols according to the length of the strip:

### 18 cm strip protocol:

-rehydration: 30 min at 20°C  
-IEF: 18°C  
-S1: step-n-hold 30 V, 10.0 hours  
-S2: step-n-hold 200 V, 1.5 hours  
-S3: gradient 3500 V, 2.5 hours  
-S4: step-n-hold 3500 V, 2.0 hours  
-S5: gradient 8000 V, 1.5 hours  
-S6: step-n-hold 8000 V, 6.0 hours

### 7 cm strip protocol:

-rehydration: 30 min at 20°C  
-IEF 18°C  
-S1: step-n-hold 30 V, 10.0 hours

IEF was stopped after 75000- 90000 V/h.

Second dimension electrophoresis was performed using a Protean II apparatus (Bio-Rad). Strips were soaked first in Equilibration buffer (EB: 6 M urea, 3% SDS, 375 mM Tris pH 8.6, 30% glycerol, 2% DTE), then in EB containing 3% iodoacetamide (IAA) and traces of bromophenol blue (BBP). Subsequently, strips were applied onto 10%-12% PA gels.

## Results

### First experimental hypothesis

As described in the Introduction section, HMGB1 is normally present in the nucleus of macrophages and translocates into the cytosol after hyper-acetylation is induced by inflammatory signals (Bonaldi et al 2003). This finding does not explain how HMGB1 can translocate across the plasma membrane. HMGB1 does not possess a leader sequence and for this reason can not enter the normal exocytotic pathway (endoplasmic reticulum/Golgi apparatus) but it is inside secretory lysosomes in macrophages (Gardella et al 2002).

Few proteins share with HMGB1 this behavior; one of these is the pro-inflammatory cytokine IL-1beta. It was shown by electron microscopy that HMGB1 and IL-1beta co-localize inside the secretory lysosomes (Gardella et al 2002); pharmacological analysis suggested the implication of ABCA1 in the process of IL-1beta exocytosis in human primary macrophages (Hamon et al 1997).

My supervisor and I hypothesized the implication of the ABC transporters also in HMGB1 exocytosis, and to demonstrate this I utilized at first two different experimental approaches, a pharmacologic and a genetic one (gain of function and loss of function assays).

### Pharmacological dissection of the ABC transporter superfamily

I tested the ability of many drugs, which were previously described to have differential inhibitory activity on various ABC transporter sub-branches (Hamon et al 1997, Wein et al 2004, Ferreria et al 2005, Ferreira et al 2005bis, Marty et al 2005, Hamilton et al 1993), in blocking HMGB1 exocytosis.

The drugs I used and their corresponding ABC transporter inhibitory activity (reported in several but not fully exhaustive publications) are ;

Glybenclamide, Ethacrinic Acid: All branches (Nofer et al 2006; Becq et al 1997; Silva et al 2004; O'Connell et al 2004; Conseil et al 2005; Kalin et al 2004; Wheeler et al 2000; Ji et al 2004)

DIDS: ABCA; ABCD; ABCC(SUR); ABCG; ABCF; ABCE (Reddy et al 2002; Xia et al 2005; Marty et al 2005; Becq et al 1997; Hamon et al 1997)

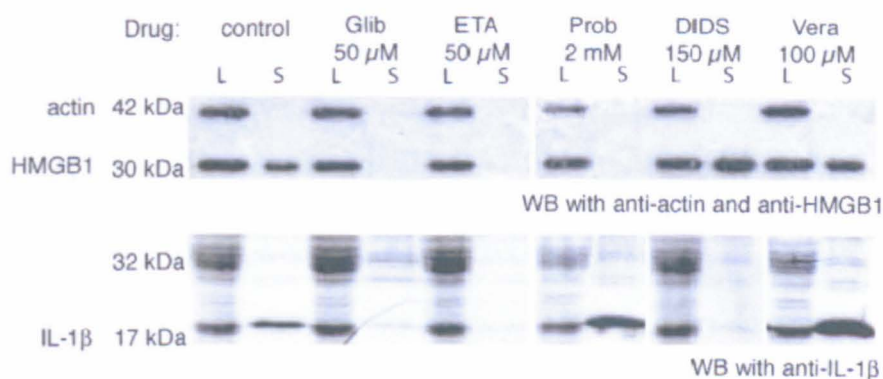
Verapamil: ABCB (Ferreira et al 2005a; Ferreira et al 2005b; Griffin et al 2005; Roman et al 2001)

Probenecid: ABCC (Webster et al 2002; Michot et al 2006; Lucia et al 2005; Jorajuria et al 2004; Olson et al 2002; Ferreira et al 2005a)

### Analysis of cell supernatants

Each drug was tested on THP-1 cells for its ability to block HMGB1 release. THP-1 is a human pro-monocytic cell line that for this experiment was differentiated into macrophagic phenotype by PMA treatment and then LPS stimulated (figure 14 upper panel). As a control I tested for the release of IL-1beta in the presence of the same drugs (figure 14 lower panel).

The protein release in THP-1 cell supernatants was analyzed by western blotting, on samples precipitated with acetone. Absence of drug-induced cell necrosis was tested by detection of beta-actin or pro-IL-1beta in the supernatants.



#### **14) Activity of ABC transporter inhibitory drugs on HMGB1 and IL-1beta release**

The upper panel shows anti HMGB1 and anti beta-actin western blots on lysates (L) and supernatants (S) of THP-1 cells treated with the different drugs. No actin is detectable in the supernatants. HMGB1 release is blocked by Glib (HMGB1), ETA (ethacrynic acid) and Prob (probenecid), DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid disodium salt) and Vera (verapamil) have no inhibitory effect.

The lower panel shows anti IL-1beta western blots on lysates (L) and supernatants (S) of THP-1 cells treated or untreated with the different drugs. No cytosolic pro-IL-1beta is detectable in the supernatants. IL-1beta release is blocked by Glib (HMGB1), ETA (ethacrynic acid) instead Vera and Prob have no inhibitory effect.

This experiment was repeated 3 times.

### Immunofluorescence analysis

Figure 15 shows an immunofluorescence against HMGB1 on mouse primary macrophages LPS stimulated in presence or absence of the different drugs.

Glybenclamide, ETA and Probenecid inhibited HMGB1 loading inside macrophagic secretory lysosomes; on the contrary DIDS and Verapamil did not show any effect.

The immunofluorescence results confirm the western blots.



### 15) Activity of ABC transporter inhibitory drugs on the loading of HMGB1 inside secretory lysosomes

Immunofluorescence images show anti-HMGB1 (red) in wt mouse primary macrophages (nuclei are counterstained blue). Macrophages were activated with 2  $\mu$ g/ml LPS in presence or absence of the different inhibitors for 18 hours. Glib, ETA and Prob block HMGB1 loading into vesicles. DIDS and Vera did not show effect. This experiment was repeated 5 times isolating macrophages from healthy mice. Scale bar is 8 $\mu$ m.

The graph shows the intersection of sets ABC transporters inhibited by the different drugs used in this study. Glib/ETA inhibit all the superfamily branches (from A to G). DIDS blocks the A, D, E, F and G branches. Prob inhibits the B branch. Vera inhibits the C (MRP and Sur) branches.

I have drawn an Euler-Venn's diagram intersecting the different sets of ABC transporters inhibited by each drug (figure 15). The diagram output indicates the probable implication of the ABCC/MRP sub-branch in the process of active exocytosis of HMGB1.

### Genetic interactions between MRPs and HMGB1

The previous experiments have shown the implication of MRP transporters in HMGB1 exocytosis. To investigate in more detail the process, and to avoid errors due to side effects of the drug utilized, I used two genetic approaches, "gain of function" and "loss of function".

#### Gain of function

The gain of function assay is based on the observation of an additional ability in cells in which the level of expression of a molecule of interest is modified.

The experimental hypothesis is that the overexpression of the different MRP/MDR molecules on the plasma membrane of "low-transporting" cells will allow them to release an increased amount of HMGB1 in the external environment.

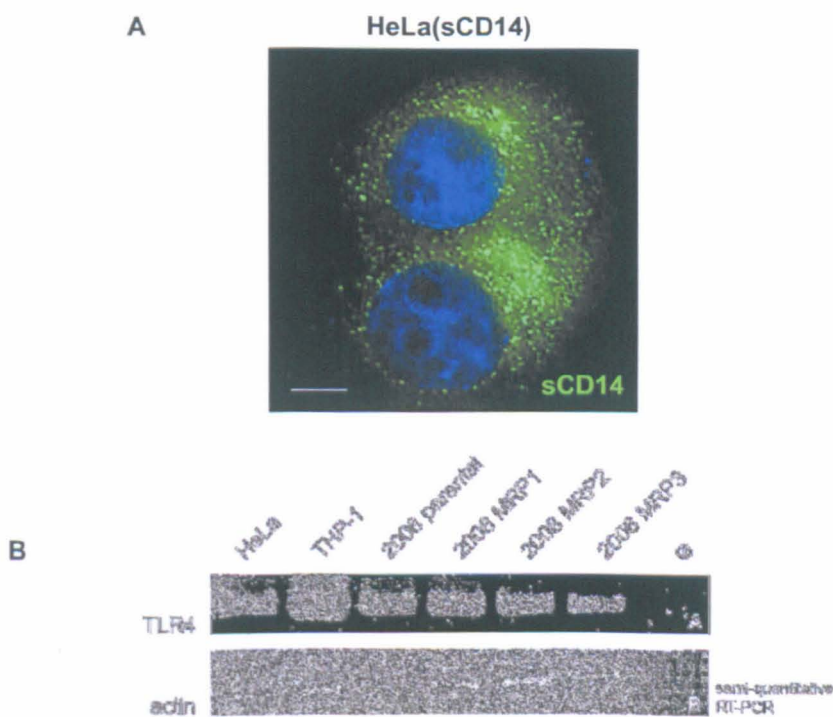
The experiment can be divided in two steps;



1) Generation of a stable HeLa cell line expressing sCD14 constitutively (figure 16A), and therefore releasing this molecule in the medium. In this cell line, TLR4 activation and HMGB1 nucleus/cytosol translocation can be triggered by addition of LPS and LPS binding protein (LBP) in the medium (Backhed et al 2002).

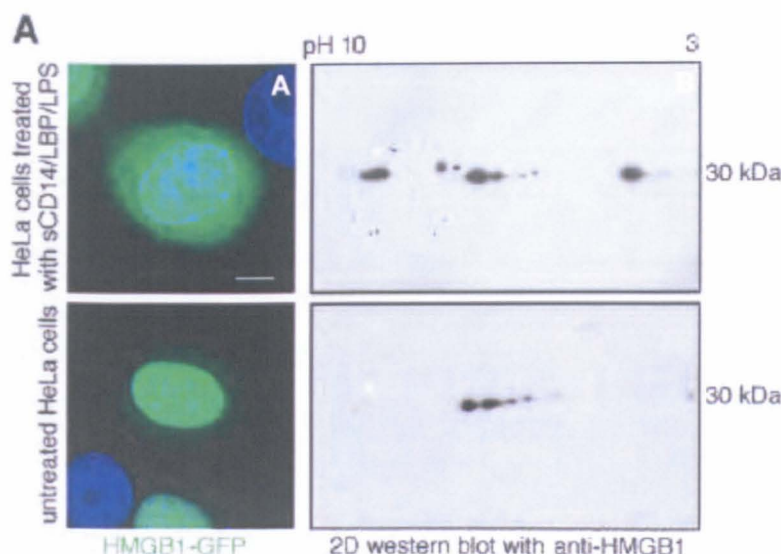
Figure 17A shows that, in HeLa cells, HMGB1-GFP translocates from the nucleus to the cytosol in the presence of the molecular complex sCD14/LBP/LPS, which specifically activates the TLR4 pathway. It is possible to see the same effect using macrophage conditioned medium plus LPS.

Figure 17B shows that in HeLa cells, after TLR4 activation, HMGB1 obtains the correct biochemical modification after nucleus/cytoplasm translocation. In the 2D western blotting, an additional HMGB1 spot appears, shifted in isoelectric point towards the acid pole, as described by Bonaldi et al in 2003.



**16) A. sCD14 overexpression in HeLa cells** Immuno-fluorescence with anti-CD14 (green) on a stable HeLa clone overexpressing sCD14. sCD14 is expressed in the ER, Golgi apparatus, and secretory vesicles. The nucleus is counterstained blue. Scale bar is 8µm.

**B. TLR4 expression.** In the upper panel, semi-quantitative RT-PCR shows TLR4 expression in all cells used. Total RNA from HeLa, THP-1 and 2008 (parental, MRP1, MRP2 or MRP3) cells was analyzed. In the lower panel the level of beta-actin is shown.



**17) A. HMGB1 cytosol-nucleus translocation after TLR4 activation** Fluorescence images show HMGB1-GFP translocation from the nucleus to the cytoplasm when the TLR4 pathway is activated in HeLa cells. Scale bar is 2.5 $\mu$ m.

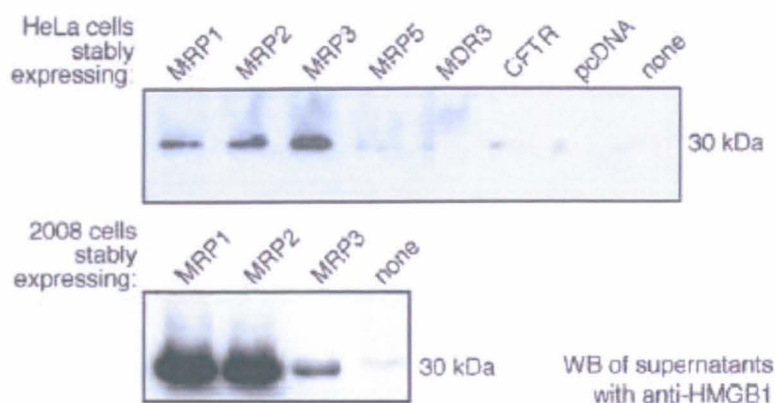
**B. TLR4 activation induces HMGB1 hyperacetylation** On the right of the fluorescence pictures the respective 2D western blot with anti-HMGB1 is shown (about 500  $\mu$ g of total protein). After TLR4 activation an additional HMGB1 spot appears, at an isoelectric point shifted towards the acidic pole (asterisk).

2) Selective overexpression of the various ABC transporters in cell lines expressing TLR4 (Figure 16B), and subsequent test for HMGB1 exocytosis.

At this point I performed the gain of function experiment (Figure 18) and I noted that overexpression of MRP1, MRP2 and MRP3 allows HeLa cells to release high amounts of HMGB1 (Figure 18 upper panel) after TLR4 activation. The same TLR4 activation experiment was performed using 2008 human ovarian carcinoma cell lines overexpressing MRP1, MRP2 and MRP3 (Figure 18 lower panel) (Hooijberg et al 2003), and the result is similar to that obtained using HeLa cells.

In HeLa-derived cell lines the membrane localization and overexpression of ABC transporters plasma was tested by immunofluorescence (as described in Materials and Methods section). I did not test for the actual functional transporters; this caveat means that the transporters that do not appear to translocate HMGB1 into the external milieu may simply be not functional despite overexpression.



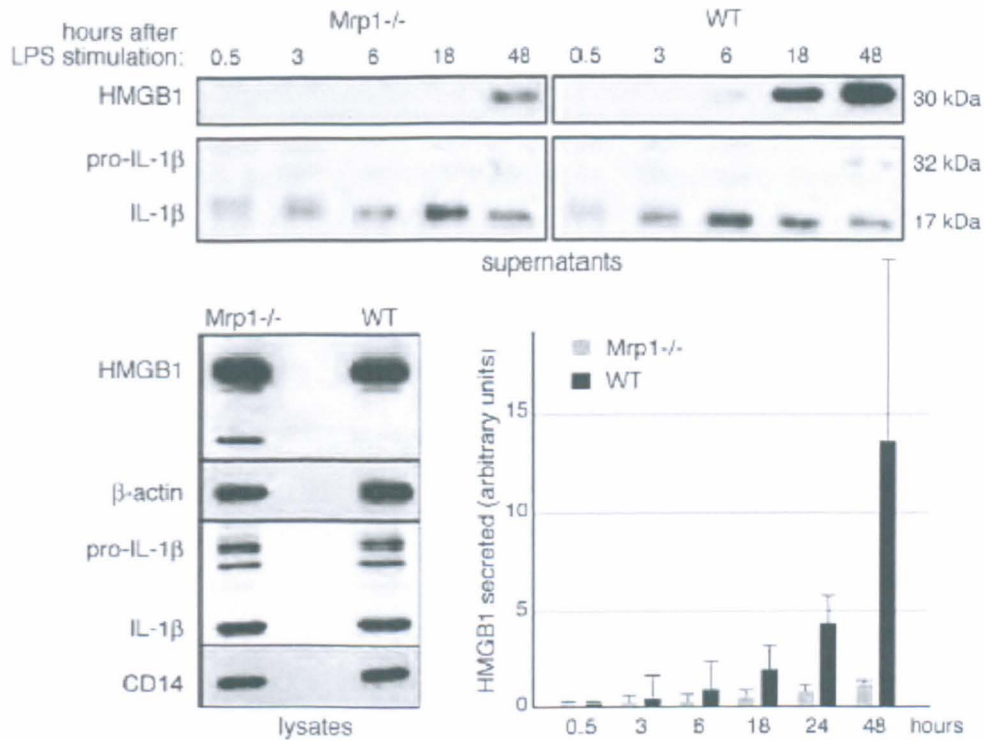


**18) Gain of function experiment** The figure shows western blotting with anti HMGB1 of supernatants of HeLa cells (upper panel) and 2008 human ovarian carcinoma cells (lower panel) overexpressing different ABC transporters. Cells that overexpress MRP1, MRP2 and MRP3 acquire the capability to secrete HMGB1. The HeLa cells experiment was repeated 5 times testing #2 clones for each construct. The 2008 cells experiment was repeated two times.

### Loss of function

The second genetic approach I utilized to investigate MRP/HMGB1 interaction is shown in figure 19. Macrophages were isolated from *Mrp1*<sup>-/-</sup> and wt mice and then stimulated with LPS; at different time points supernatants were tested for HMGB1 release by western blotting with anti-HMGB1 antibodies. As expected, macrophages without the transporter molecule fail to release HMGB1 with high efficiency, if compared with the wt at the some time points. IL-1 $\beta$  is released by both *Mrp1*<sup>-/-</sup> and wt macrophages, so secretory lysosomes have no degranulation defect. The absence of cell necrosis was tested by assaying for pro-IL-1 $\beta$  in the supernatant.

This experiment was repeated 5 times, both monitoring HMGB1 secretion from macrophages obtained from single individuals, and from pools of several individuals, with similar results. Figure 19 shows the time course of HMGB1 secretion. Statistical analysis (non-parametric Kruskal-Wallis ANOVA) shows the difference between *Mrp1*<sup>-/-</sup> and wt macrophages to be highly significant ( $P < 0.01$ ). However, it should be noted that, although MRP1 appears to be the major HMGB1



### 19) Loss of function in Mrp1 $^{-/-}$ macrophages

The upper panel shows a western blot anti HMGB1 and anti IL-1-beta on Mrp1 $^{-/-}$  and wt macrophage supernatants, collected at different time points after LPS stimulation. IL-1beta is released by both cell types, although with slightly different kinetics. Pro-IL-1beta is used as cell necrosis marker and is detected in the supernatants after 2 days.

The histogram shows means and standard deviations of the amount of HMGB1 released by 500 000 Mrp1 $^{-/-}$  and wt macrophages at different time points after LPS stimulation. Each value is the average of results coming from 5 different experiments:

- 1) Pool of 3 male and 2 female Mrp1 $^{-/-}$  mice vs matched wt mice
- 2) Mrp1 $^{-/-}$  female 2D vs wt female
- 3) Mrp1 $^{-/-}$  female 3F vs wt female
- 4) Mrp1 $^{-/-}$  female 3E vs wt female
- 5) Pool of 3 males Mrp1 $^{-/-}$  mice vs matched wt mice

Quantification was done by densitometry on western blots with anti HMGB1 using the ImageJ program. Values were normalized against HMGB1 released by wt macrophages after 30 minutes, which was put equal to 1. Samples were analysed also for the presence of pro-IL-1beta (32 kDa), marker of cell necrosis, and IL-1beta (17 kDa) marker of normal secretory lysosomes degranulation. Statistical analysis (ANOVA Kruskal-Wallis two ways) shows that the differences between wt and Mrp1 $^{-/-}$ -macrophages is highly significant ( $p < 0.01$ ).

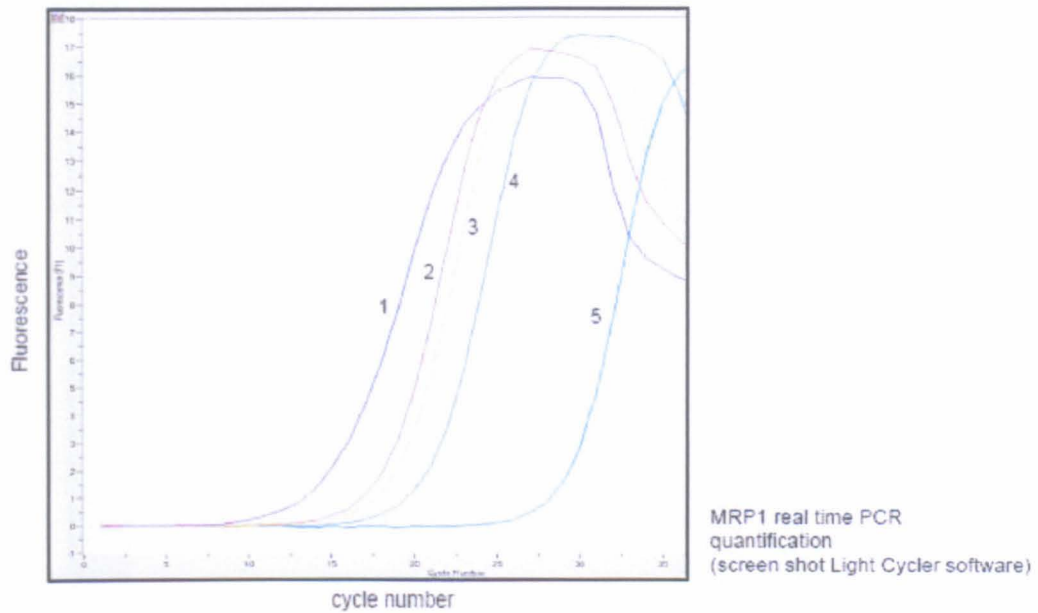
The lower left panel show western blots on whole macrophage cell lysates (Mrp1 $^{-/-}$  and wt), collected after 18 hours of stimulation. The filters were probed with anti HMGB1, anti IL-1beta, anti beta-actin and anti-CD14 antibodies. HMGB1 is present also in a faster migrating form in Mrp1 $^{-/-}$  macrophages. Beta-actin was used as a loading control.

transporter in mouse macrophages, most likely it is not the only one: a small amount of HMGB1 is also released by *Mrp1*<sup>-/-</sup> macrophages.

Western blots with anti CD14, IL-1 $\beta$ , HMGB1 and beta-actin on cell lysates collected after 18 hours of stimulation show no detectable differences between *Mrp1*<sup>-/-</sup> and wt macrophages, except for the appearance of a faster migrating HMGB1 form (perhaps a degradation product) in *Mrp1*<sup>-/-</sup> macrophages.

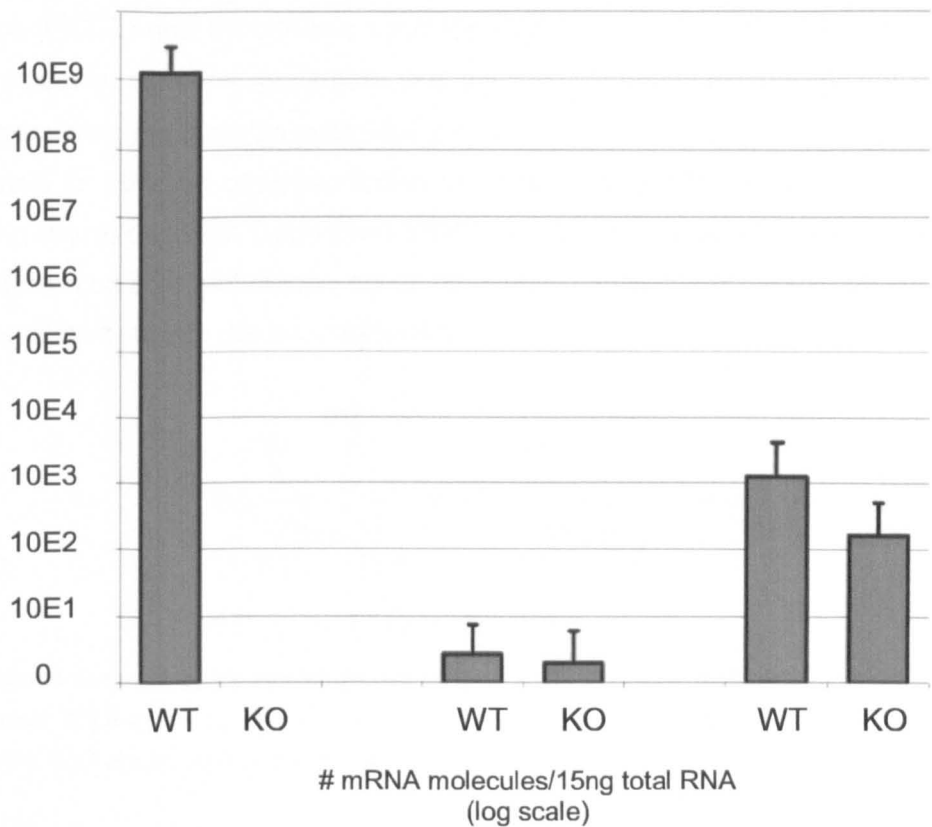
### **MRP expression level in mouse peritoneal macrophages**

In order to evaluate the expression level of MRP1, MRP2 and MRP3 in peritoneal macrophages, I performed real time PCR using the LightCycler (Roche) technology on cDNAs obtained from reverse-transcription of total RNA isolated from *Mrp1*<sup>-/-</sup> and wt macrophages. I used primers specific for mouse MRP1, MRP2 and MRP3 cDNAs. Known template quantities (linearized plasmids containing MRPx coding sequences) were used to draw a titration curve. Figure 20 shows the LightCycler screen shot of one explicative quantification reaction. LightCycler is able to measure fluorescence intensity pattern (sigmoid) during the PCR cycling and this is directly proportional to the DNA quantity in each sample. PCR were performed on samples where the number of cDNA molecules was known (standard) or unknown (total RNA) and the software is able to draw a titration curve using as reference the given standard points, making a correlation between fluorescence intensity and the number of cDNA molecules.



- 1) standard  $10E+15$  MRP1 molecules (plasmidic cDNA)
- 2) standard  $10E+10$  MRP1 molecules (plasmidic cDNA)
- 3) WT 15ng totRNA
- 4) standard  $10E+5$  MRP1 molecules (plasmidic cDNA)
- 5) KO 15ng totRNA

**20) Real time PCR** The graph shows the fluorescence intensity pattern during PCR reactions which were performed on samples where the number of MRP1 cDNA molecules was known (standard) or unknown (total RNA). The software is able to draw a titration curve using as reference the standard points, making a correlation between fluorescence intensity and the number of cDNA molecules.



**21) MRP1, MRP2 and MRP3 mRNA level in mouse peritoneal macrophages wt and Mrp1-/-** This panel shows the quantification of mRNAs coding for MRP1, MRP2, MRP3 in mouse peritoneal macrophages (WT and Mrp1-/-). Data were collected from several experiments performed on totRNA derived from different macrophage isolations. MRP1 is expressed at extremely high level in comparison with MRP2 and 3.

The results (Figure 21) indicate a significance expression for MRP1 in wt macrophages, and the absence of MRP1 expression in Mrp1-/- macrophages, as expected. MRP2 and MRP3 are expressed at low levels, both in wt and Mrp1-/- macrophages.

Thus, even if MRP2 and MRP3 can transport HMGB1, most secretion in macrophages would occur via MRP1. This is in accordance with the results obtained with macrophages from Mrp1-/- mice.

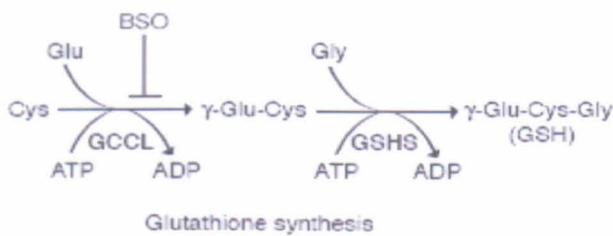
**MRP1/HMGB1 interaction and glutathione modification**

MRP1 is an ABC transporter characterized for its ATP dependent pump activity on GSH modifide compounds. GSH modification (GS-X) is also called glutathionylation and glutathione is an enzymatically synthesized tripeptide (Figure 23) which is used to maintain the intracellular redox potential, and acts as “garbage label” to identify toxic compounds.



GSH is synthesized in a series of biochemical reactions catalyzed by cysteine-glutamate ligase (GCCL) and Glutathione synthase (GSHS) which require ATP, magnesium and three amino acids: cysteine, glutamate and glycine. Glutathione binds its substrates by disulfide bridges using the cysteine sulfhydryl moiety.

I tested for HMGB1 glutathionylation "in vitro" and "in vivo" (figures 24-29) by the fact that during my experiment I observed MRP1 playing its transporter activity onto HMGB1 and I hypothesized that glutathione could be a post-translational modification .necessary for the recognition between the two molecules.



**23) Glutathione de novo synthesis** Glutathione is composed of three amino acids, Glu, Cys and Gly. Its synthesis is catalyzed by two ATP dependent enzymes, Glu-Cys ligase (GCCL) and Glutathione synthase (GSHS). BSO blocks GSH synthesis acting on GCCL.

I assayed for the presence of GSH covalently bound to HMGB1 using two approaches:

- 1) HMGB1 reactivity to an antibody specific for GSH bound to protein (Figure 24)
- 2) radiolabeling the GSH/GSSG intracellular pool, and testing whether HMGB1 became radioactive (Figure 25).

HMGB1 glutathionylation assay using anti-GSH antibody

Figure 24 shows HMGB1 reactivity to anti-GSH antibody. HMGB1 was immunoprecipitated, in non reducing conditions, from THP-1 whole cell lysate, after treatment with PMA and LPS stimulation.

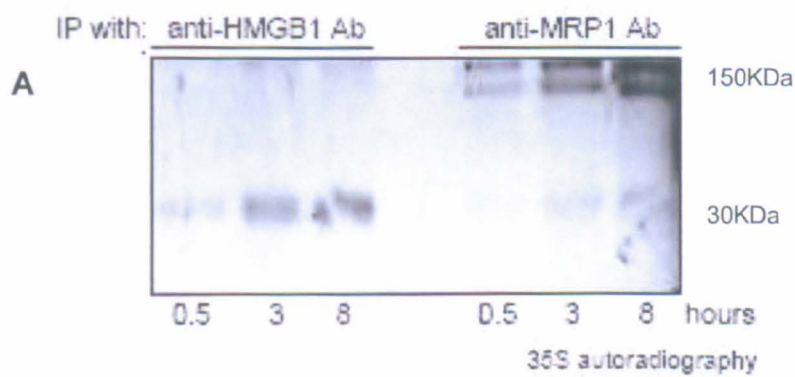


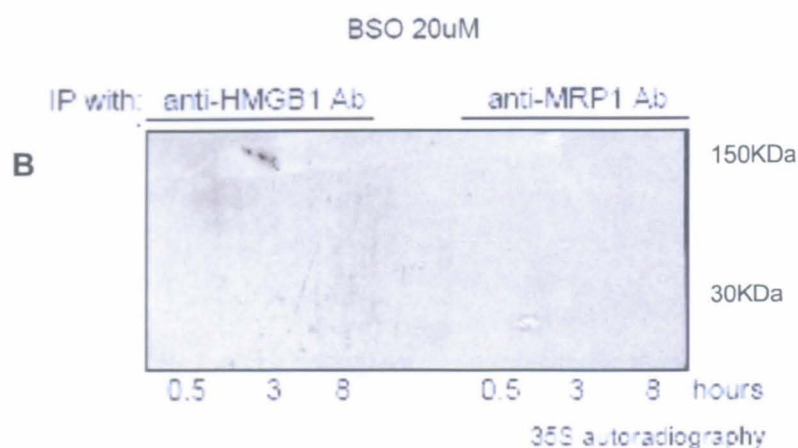
**24) Detection of HMGB1 glutathionylation using an anti-glutathione antibody** HMGB1 was immunoprecipitated from THP-1 cells differentiated to macrophages and LPS-stimulated for 18 hours. The immunoprecipitated material was loaded in reducing (DTT) or non-reducing conditions on non-reducing SDS-PAGE, and immunodetected with anti-HMGB1 and anti-GSH. HMGB1 is present in two different forms with different electrophoretic mobility, the slower form is recognized by the anti-GSH antibody.

Samples were resolved by SDS-PAGE in non-reducing conditions and then probed with anti-HMGB1 and anti-GSH antibodies; as control for the signal specificity reducing conditions were restored adding 20 µM DTT. In non-reducing conditions HMGB1 displays two different forms(apparent molecular weight 35KDa and 28KDa), one of which is recognized by the anti-GSH antibody.

HMGB1 glutathionylation assay by GSH/GSSG intracellular radiolabelling

The second experimental approach I utilized to investigate HMGB1 glutathionylation was the radiolabelling of the GSH/GSSG intracellular pool. I gave a <sup>35</sup>S-cysteine pulse to THP-1 cells differentiated to macrophages and activated with LPS, in the presence of protein synthesis block (cycloheximide) (see Materials and Methods). In these conditions radiolabelled glutathione can be synthesized *de novo*, since its biosynthetic pathway does not require ribosomal activity (Figure 23).





**25) GSH/GSSG intracellular radiolabelling** THP-1 cells differentiated to macrophages were stimulated with LPS. Protein synthesis was blocked with cycloheximide and GSH/GSSG intracellular pool was labeled with a  $^{35}\text{S}$ -Cys pulse, in the presence (B) or the absence (A) of the GCCL inhibitor BSO. Samples were collected at different time points after the beginning of LPS stimulation, and immunoprecipitated with anti-HMGB1 and anti-MRP1. The immunoprecipitated material was resolved by SDS-PAGE in non-reducing conditions. This experiment was repeated 2 times.

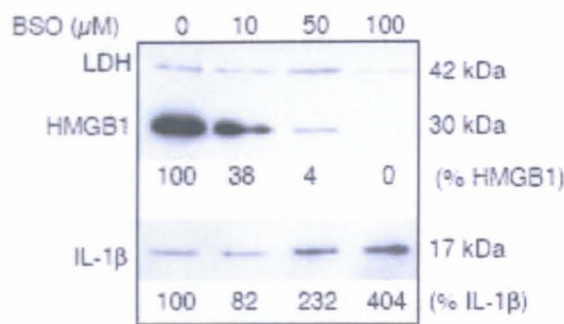
HMGB1 and MRP1 were immunoprecipitated at different time points after the beginning of LPS stimulation. Samples were electrophoresed in non-reducing conditions and the incorporated radioactivity was measured by autoradiography. An increasing amount of radioactivity was associated to both HMGB1 and MRP1 with time (Figure 25a).

GCCL catalytic activity can be inhibited by L-buthionine-sulfoximine (BSO), which binds covalently to a free thiol in the active site. Pretreatment with 20  $\mu\text{M}$  BSO blocks HMGB1 and MRP1 radiosignal incorporation (Figure 25b).

#### HMGB1 secretion after blockage of glutathione synthesis

To test whether glutathione is necessary for HMGB1 release, I depleted the GSH/GSSG intracellular pool. I pre-treated THP-1 cells differentiated to macrophages with increasing BSO concentrations (from 0 to 100  $\mu\text{M}$ ) for 8 hours before LPS stimulation. Supernatants were collected 18 hours after LPS stimulation. I tested for drug induced cell necrosis with anti-LDH antibody, and for IL-1 $\beta$  and HMGB1 secretion. IL-1 $\beta$  release is enhanced in the presence of BSO, as reported by Haddad in 2002. HMGB1 release is strongly impaired (Figure 26).





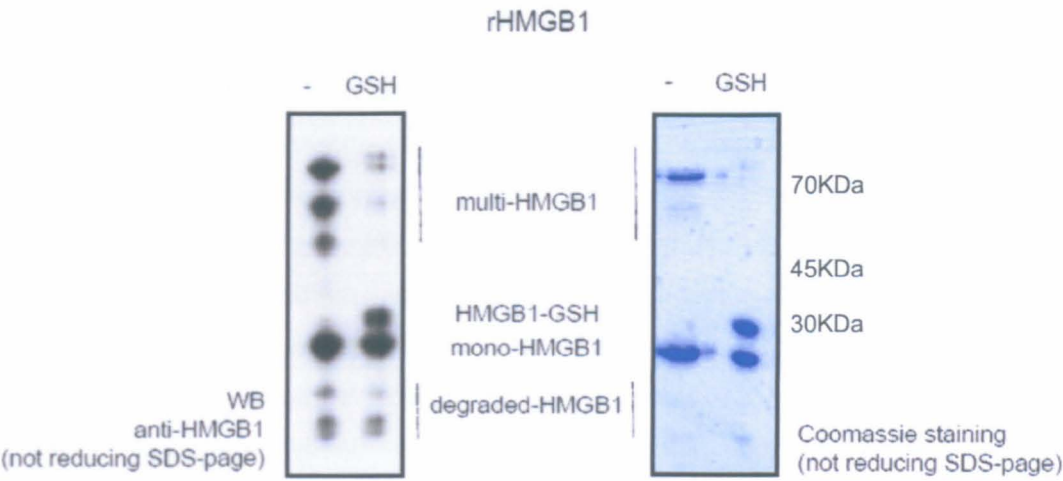
**26) BSO inhibits HMGB1 secretion** Western blot on supernatants of THP-1 cells differentiated to macrophages and pre-treated with BSO before LPS activation. The filter was probed with anti-LDH antibody, to detect drug induced cell necrosis, with anti-HMGB1 and anti-IL-1beta antibodies, to test for the amount of protein secretion. This experiment was repeated 2 times.

In vitro glutathionylation of recombinant HMGB1

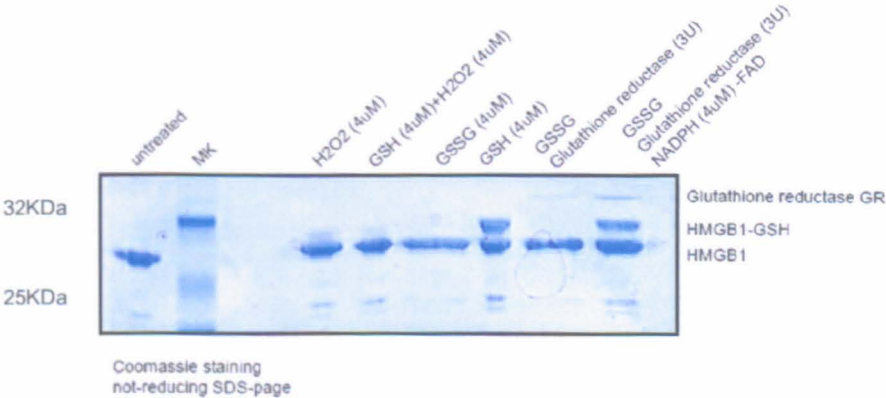
To further understand the HMGB1 translocation mechanism, I investigated if HMGB1 glutathionylation is a spontaneous process driven by redox potential or if it is an enzyme-catalyzed reaction.

Indeed, HMGB1 can be glutathionylated *in vitro* by incubating the recombinant protein (rHMGB1) in the presence of reduced glutathione (GSH). Figure 24 shows that recombinant HMGB1 migrates in non-reducing SDS-PAGE as a series of bands (whereas it migrates as a single band in reducing conditions, not shown here). We infer that the slower running bands represent HMGB1 multimers, deriving from the formation of SS bonds between HMGB1 monomers. After incubation with reduced glutathione (GSH), the putative HMGB1 multimers disappear, and a new band appears, migrating just above the unmodified HMGB1 monomer (apparent molecular weight 28KDa).

The slowly migrating HMGB1 band is not produced after incubation of rHMGB1 with oxidized glutathione (GSSG), but is evident if HMGB1 and GSSG are incubated with glutathione reductase and NADPH, which reduce GSSG to GSH (Figure 26).



27) Recombinant HMGB1 reacts with reduced glutathione in vitro Coomassie staining and Westewn blot with anti-HMGB1 antibodies of recombinant HMGB1 (500 ng) before and after incubation with 4  $\mu$ M GSH, resolved in non-reducing SDS-PAGE.



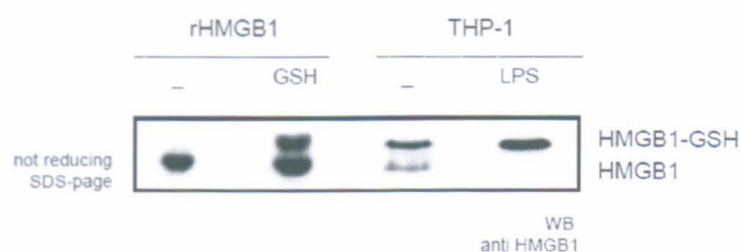
28) Recombinant HMGB1 reacts with oxidized glutathione only in the presence of glutathione reductase and NADPH Coomassie staining of non-reducing SDS-PAGE loaded with recombinant HMGB1 (500 ng) after incubation with the indicated reactants:

- Hydrogen peroxide 4  $\mu$ M
- GSH 4  $\mu$ M + hydrogen peroxide 4  $\mu$ M
- GSSG 4  $\mu$ M
- GSH 4  $\mu$ M
- GSSG + glutathione reductase w/o electron donor
- GSSG + glutathione reductase + NADPH

From these experiments I infer that HMGB1 reacts spontaneously with reduced GSH. However, this does not exclude that a catalyzed reaction might occur inside the cell.

### Comparison between *in vitro* and *in vivo* glutathionylated HMGB1

In western blots after non-reducing SDS-PAGE, anti-HMGB1 antibodies recognize two HMGB1 forms with different electrophoretic mobility; the slower band is also recognized by the anti-GSH antibody in the immunoprecipitation assay (figure 24). Figure 29 shows that, in THP-1 cells differentiated to macrophages, the slower band recognized by anti-HMGB1 antibodies migrates with the same mobility of HMGB1 after incubation with reduced GSH; we therefore identify it as glutathionylated HMGB1. The faster band has the same electrophoretic mobility as unmodified HMGB1. In unstimulated cells, the amount of glutathionylated HMGB1 is already significant; after stimulation of the cells with LPS, almost all HMGB1 is found in glutathionylated form.



**29) Glutathionylation of recombinant and endogenous HMGB1** Western blot with anti HMGB1 antibodies after SDS-PAGE in non-reducing conditions. The gel was loaded with 0.5  $\mu$ g of recombinant HMGB1 treated or untreated for 5 minutes with 4  $\mu$ M reduced glutathione and whole lysates of 500 000 THP-1 cells differentiated to macrophages, stimulated with LPS or not for 18 hours. This experiment was repeated 2 times.

### **HMGB1 cysteine residues**

A possible target for the formation of disulfide bridges with glutathione are cysteine residues. HMGB1 has three cysteine residues (number 22, 44 and 105) (figure 30a). I decided to abolish these thiols by mutating the 3 cysteines into serines. I generated 3 single cysteine to serine mutants, C22S, C44S and C105S, and one triple cysteine to serine mutant C22/44/105S. I transfected HeLa cells with wt and mutant forms of HMGB1 in fusion with GFP (Figure 30c) and I analyzed the subcellular localization of these proteins in normal conditions and after TLR4 activation.

In unstimulated conditions, mutant proteins are expressed and have a nuclear localization like wt HMGB1, and after TLR4 activation translocate from the nucleus to the cytosol.



HMGB1 cDNA sequence

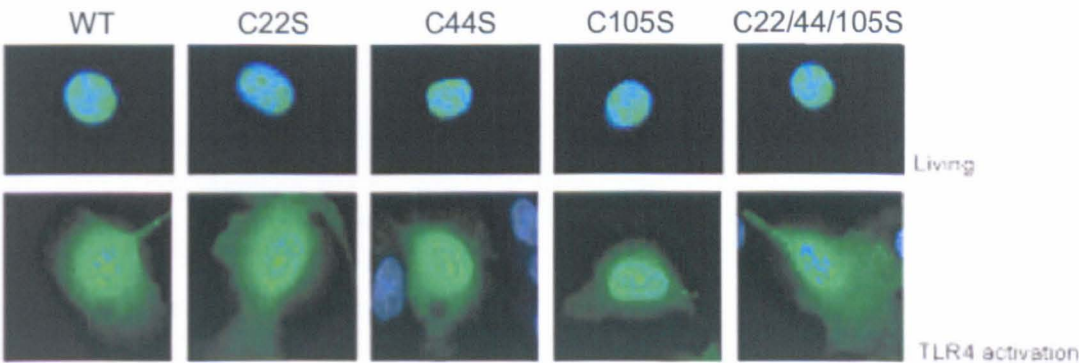
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M G K G D P K K P R G K M S S Y A V V V
caa acc cgg cgg gag gag cag aag aag aag cac cag gat gct tct gtc aac ttc tca gag
Q T R E E H K K K H P D A S V N P S E
ttc tcc aag aag tca gag agg tgg aag acc atg tct gct aaa gaa aag ggg asa ttt
F S K K S E R W K T M S A K E K G K P
gaa gat atg gca aag gct gac aag aag gct ogt tat gaa aga gaa atg aaa acc tac atc ccc
E D M A K A D E A R Y E R E M K T Y I P
ccc aaa ggg gag acc aaa aag aag ttc aag gac ccc aat gcc ccc aag agg cct cct tgg
P K G E T K K K P K D P H A P K R P P S
gcc ttc ttc ttg ttc tct gag tac ogc cca aaa atc aaa ggc gag cat cct gcc tta
A P P L P S W Y R P K I K G E H P G L
tcc att ggt gat gtt ggg aag aaa cta gga gag atg tgg aac aac act gct gcg gat gac
S I G D V A K K L G R M W W H T A A D D
aag cag ccc tat gaa aag aag gcc gcc aag ctg aag gag aag tat gag aag gat att gct
K Q P Y E K K A A K L K E K Y E K D I A
gcc tac aga gct aaa gga aaa cct gat gca gcg aaa aag ggg gtg gtc aag gct gag aag
A Y R A K G K P D A A K K G V V K A R K
agc aag aaa aag aag gaa gag gaa gac gag gag gat gaa gag gat gag gaa gag gag
S K K K E E E D D E E D E E D E R R R
gaa gag gag gaa gac gaa gat gaa gaa gaa gat gat gat gat gaa taa
E E E E D E D W E E D D D D E -
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A

Hmgb1-/- (MRP1 overexpressing) cells



B



C

30) HMGB1 cysteine mutants

A. HMGB1 (*Rattus norvegicus*) nucleotide and amino acid sequences. In green, cysteine residues.

B. Western blot with anti-HMGB1 of whole cell lysates supernatants of Hmgb1-/- fibroblasts expressing different mutant or wt HMGB1. Samples were collected after TLR4 activation (18 hours). Wt and C22S HMGB1 are

normally released, instead C44S, C105S and C22/44/105S are not detectable. Fibroblasts were transiently cotransfected with MRP1 overexpressing construct (experiment was repeated 2 times).

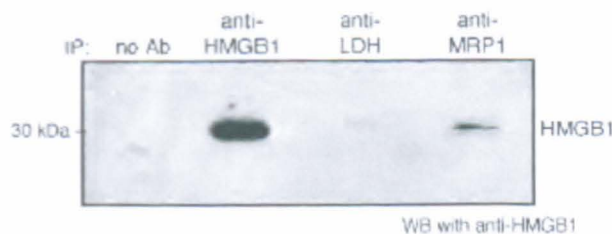
**C.** HeLa cells transfected with wt, C22S, C44S, C105S and C22/44/105S HMGB1 in fusion with GFP (in green). In normal unstimulated conditions mutant proteins and wt are nuclear, after TLR4 activation (18 hours) they are partially cytosolic. Nuclei are counterstained blue with Hoechst 33342. Scale bar is 8µm. The experiment was repeated 2 times.

To test whether the HMGB1 mutants can be secreted via MRP1, I then cotransfected Hmgb1<sup>-/-</sup> fibroblasts with the MRP1 expression construct (pcDNA-MRP1) and wt or mutant HMGB1 vectors. I then stimulated these cells with LPS/CD14/LBP and tested the supernatants for secreted HMGB1 (wt or mutant). The triple mutant C22/44/105S, and the single mutants C44S and C105S cannot be detected in the supernatant, instead the C22S mutant and wt HMGB1 are normally secreted (figure 30b).

### MRP1 and HMGB1 molecular interaction

#### MRP1-HMGB1 co-immunoprecipitation

If HMGB1 traverses lipid membranes across MRP1, there might exist a quite stable if transient interaction between HMGB1 and MRP1. I tried a co-immunoprecipitation assay using the anti-MRP1 monoclonal antibody QCRL-3, which recognizes an epitope on the intracellular side of the transporter in native conditions; it does not recognize the denatured epitope (Hipfner et al 1999). Remarkably, a faint but distinct band of HMGB1 was detectable in the QCRL-3 immunoprecipitate. Control immunoprecipitations with no antibody, or an irrelevant antibody against lactate dehydrogenase, recovered no HMGB1 (figure 31).

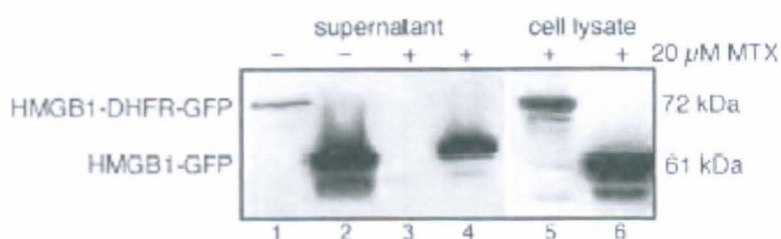


**31) MRP1 and HMGB1 co-immunoprecipitation** Whole lysates of THP-1 differentiated to macrophages, after 18 hours LPS activation, were immunoprecipitated with the indicated antibodies. The beads were then boiled in SDS-PAGE loading buffer, electrophoresed, and western blotted with anti-HMGB1.

### Unfolding assay

HMGB1 has a distinct non-globular structure (Knapp et al 2004) with 2 independent HMG boxes and a long unstructured tail. Each HMG box is composed of 3 alpha helices. I asked how it can traverse MRP1, which looks like a cylinder which has an outer diameter of 10 nm and an internal cavity of a maximum of 5 nm and an even smaller central gate (Rosenberg et al 2001). HMGB1 might sneak through the MRP1 cavity retaining its folding, or it could thread through it after reversible unfolding. Notably, HMGB1 can refold spontaneously after thermal denaturation (Ramstein et al 1999).

I generated a chimeric protein fusing HMGB1, DHFR and GFP (in that order from the N- to the C-terminus) and I tested whether it can traverse MRP1. HeLa cells overexpressing MRP1 and HMGB1-DHFR-GFP did in fact secrete the fusion protein, after LPS/sCD14/LPB stimulation, albeit a much lower level than the HMGB1-GFP fusion protein (Figure 32). Methotrexate (MTX) binds tightly to the catalytic site of DHFR and prevents its unfolding (Eilers et al 1986); in the presence of MTX, the HMGB1-DHFR-GFP fusion was retained inside the cell, indicating that its unfolding is required for transport by MRP1. The control HMGB1-GFP fusion was secreted at approximately the same level even in the presence of MTX. This experiment suggests that to be translocated in the external environment HMGB1 and all the proteins in fusion with it must be unfolded, even if, this kind of experiment is only an indirect proof of unfolding necessity of the whole reporter chimerical protein. The same experiment was performed to test FGF-2 secretion (Backhaus et al 2004).



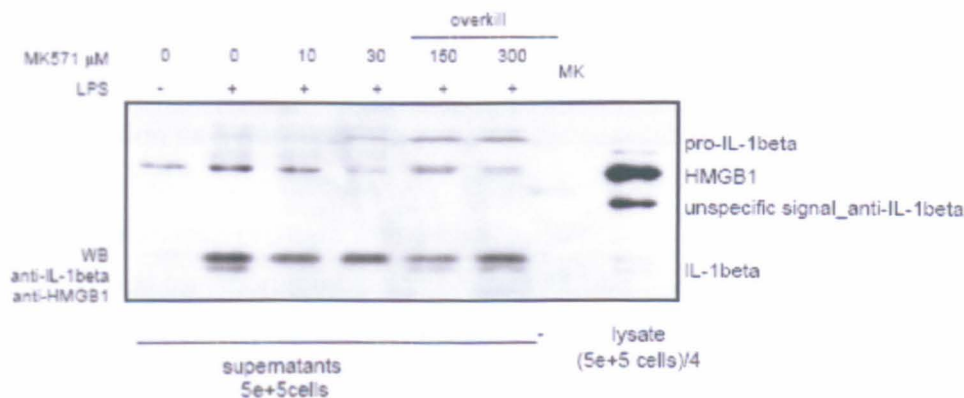
**32) Unfolding-dependent HMGB1 translocation throughout MRP1** HeLa cells overexpressing MRP1 were transiently transfected with the chimeric proteins HMGB1-DHFR-GFP and HMGB1-GFP. After TLR4 activation, in presence or absence of 20 μM methotrexate (MTX), cell supernatants and lysates were analyzed by western blotting with anti-GFP.

### MK571

The drug MK571, (E)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid, was developed in 1989 by Jones and co-workers as a specific LTC<sub>4</sub> receptor antagonist and subsequently (Jedlitschky et al 1994) it was



found that at much higher concentration ( $\mu\text{M}$  compared to  $\text{nM}$ ) it can be a specific inhibitor for MRP1 and MRP2 (Chen et al 1999, van der Kolk et al 1998), but in different conditions MK571 can also inhibits MRP3 (Li et al 2003) and MRP4 (Chen et al 2002). Here I show an experiment in which I tested for MK571 inhibitory activity on HMGB1 and IL-1 $\beta$  exocytosis (figure 30). I performed a western blotting on supernatants of THP-1 derived macrophages, LPS stimulated, in the presence of MK571 increasing concentration. HMGB1 exocytosis inhibitory concentration range is around 30  $\mu\text{M}$  and IL-1 $\beta$  exocytosis is not affected in the presence of MK571. MK571 is overkilling at concentrations over 30  $\mu\text{M}$  and it must be highlighted that this result is preliminar because of the difficulty of maintaining cells alive during MK571 treatment.



**33) MK571 and HMGB1 exocytosis** The figure shows a WB on supernatants and whole cell lysate of THP-1 derived macrophages (PMA 0,5 $\mu\text{M}$ ) stimulated with LPS (5 $\mu\text{g}/\text{ml}$ ) in presence or absence of MK571 at increasing concentration. Stimulation was performed for 18hrs. Pro-IL-1 $\beta$  release is used as index of drug induced cell necrosis. MK571 is inhibitory for both HMGB1 at 30  $\mu\text{M}$ , it is overkilling at concentrations higher than 150 $\mu\text{M}$  (pro-IL-1 $\beta$  release). This experiment was performed in duplicate.

## Discussion

HMGB1 is a bi-functional abundant nuclear protein which plays a role in transcriptional regulation but also in inflammation and tumor growth (Lotze and Tracey 2005). HMGB1 has been associated with cancer growth and dispersal: antibodies against HMGB1 and decoy receptors for it (soluble RAGE) hinder the growth of the primary tumour mass and reduce the number of metastases (Taguchi et al 2000, Huttunen et al 2002).

HMGB1 is actively released by competent cells, avoiding the canonical exocytosis route via ER/Golgi; HMGB1 does not possess a leader signal in its amino acid sequence (Nickel 2005, Degryse et al 2001, Schlueter et al 2005, Wang et al 1999).

In my thesis work I identified and characterized the molecular mechanism by which HMGB1, as a leaderless protein, can be released in the external milieu with high efficiency and in a controlled manner.

I have identified MRP transporters as the major players involved in the process, and HMGB1 glutathionylation as a necessary post-translational modification.

Identification of MRP transporters as HMGB1 carrier molecules was obtained approaching the experimental problem with different assays:

- a) Pharmacological transporter inhibition (figures 14 and 15)
- b) MRP transporter overexpression (figure 18)
- c) MRP transporter knock out (figure 19).

The different drugs I used at the beginning allowed me to dissect the ABC transporter superfamily by inhibiting specific branches, one of which is the MRP branch. I observed that relatively unspecific compounds, such as ethacrynic acid (powerful inhibitor of all the ABC transporter superfamily), more specific compounds, such as probenecid (preferentially inhibits the ABCC branch), and finally specific inhibitors, such as MK571 (a drug designed to inhibit MRP1 and MPR2) all block HMGB1 release.

Pharmacological treatments cannot fully prove direct molecular interactions between two perturbed cellular functions (MRP functionality and HMGB1 translocation), and this is due to drug side effects; for this reason I performed genetic tests of gain and loss of function. In the gain of function assay I observed that not only MRP1 but also MRP2 and MRP3 are able to translocate HMGB1 in the external environment. In the loss of function assay I observed that *Mrp1*<sup>-/-</sup> macrophages show a severe deficiency in HMGB1 secretion, but not in IL-1 $\beta$  secretion. Significantly, in macrophages MRP1 is by far the most expressed transporter of the MRP1, -2 and -3 trio (figure 19quater). However, even *Mrp1*<sup>-/-</sup> macrophages release a small amount of HMGB1.



Intriguingly, Mrp1<sup>-/-</sup> mice have an inflammation-defective phenotype, described since 1997 (Wijnholds et al) in the laboratory Piet Borst. Moreover these mice show defects in DC migration to the lymph nodes (Robbiani et al 2000). So far, the functional interpretation of the inflammation defects of Mrp1<sup>-/-</sup> mice defects was ascribed to the inability to secrete LTC<sub>4</sub>. HMGB1 is a late inflammatory mediator and is necessary for peripheral DC maturation (Dumitriu et al 2005), so the phenotype Mrp1<sup>-/-</sup> mice can in part be due to the absence of HMGB1 in some step of the inflammatory cascade, arguably in later phases.

HMGB1 and IL-1 $\beta$ , another leaderless cytokine, partially colocalize in macrophages secretory lysosomes (Gardella et al 2002), even if the exact characterization of these acidic organelles needs additional studies. In Mrp1<sup>-/-</sup> macrophages IL-1 $\beta$  loading and release is not affected, but only somewhat delayed; this underlines that IL-1 $\beta$  and HMGB1 do not utilize the same carrier molecules to traverse the membranes of secretory lysosomes, and also that degranulation of these organelles is not affected.

MRP transporters are described as gated, cylindrical, membrane embedded, hydrophilic pores (Rosenberg et al 1997), and HMGB1, which has a bi-lobar structure (Knapp et al 2004), must pass through them. I tried to give a mechanistic explanation of the process using a unfolding assay based on DHFR and MTX (Eilers and Schatz 1986), and I suggest that HMGB1 has to unfold its tertiary and secondary structure during the translocation process (figure 28).

Moreover, MRP1 and MRP2 have been known since the 80s as pumps that actively transport glutathionylated compounds (Leier et al 1994). I looked *in vivo* (figures 21 and 22) and *in vitro* (figure 24) for a possible glutathione modification of HMGB1. I found that purified recombinant HMGB1 is highly reactive to reduced glutathione, and intracellular HMGB1 appears modified by glutathione. Mutating the HMGB1 cysteine residues 44 and 105 blocks HMGB1 secretion (figure 26), which suggests that these are the GSH reactive sites. These findings suggest that MRPs can recognize HMGB1 for transport via its glutathione modification.

The ability of MRPs to transport polypeptides is not completely new. In fact, other components of the ABC transporter superfamily are known to translocate antigenic peptides originated by proteasomal degradation, namely the ABCB2/ABCB3 heterodimer (TAP1/TAP2 protein), which is expressed on the ER of phagocytic cells displaying MHC class I molecules (Vos et al 1999; 2000). MRP1 itself can transport small hydrophobic peptides (de Jong et al 2001). I now show that a full length, functional protein can be recognised and transported by MRPs.

The link between HMGB1 secretion and MRP1 throws some light on the mechanism of at least one non-classical transport pathway. In addition, it may have unexpected practical value in the management of cancer patients. I argue that MRP expressing tumour cells will secrete HMGB1 at a significant rate, with possible unfavourable consequences. Long-term tumour drug treatments could positively select MRP expressing tumour cell clones. MRPs expression by tumour cells may give them positive fitness by permitting them to secrete HMGB1: via HMGB1 tumour cells may stimulate their own cell growth and induce angiogenesis to feed themselves (Zeh and Lotze 2005, Schlueter et al 2005). In this case targeting HMGB1 secretion might be highly relevant for subsets of drug resistant cancer patients, even more so than for cancer patients in general (Lotze and DeMarco 2003), as well as for patients with sepsis.

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